METHOD FOR ATTENUATING VIRULENCE OF MICROBIAL PATHOGENS AND FOR INHIBITING MICROBIAL BIOFILM FORMATION

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The present invention relates to the use of cyclic dinucleotides to attenuate virulence of microbial pathogens and to inhibit biofilm formation, thereby controlling microbial colonization or infections caused by a wide variety of microbial species.

Description of the Related Art

[0002] Cholera is an important diarrheal disease of humans that results in significant morbidity and mortality (Pollitzer, 1959; and Kaper et al., 1995). Cholera affects more than 75 countries and every continent (Communicable Disease Surveillance and Response, World Health Organization, who.org). Cholera is acquired by drinking fecally contaminated food or water containing pathogenic *Vibrio cholerae* that can colonize the small intestine and release cholera toxin (CT) resulting in massive secretory diarrhea and death if untreated (Kaper et al., 1995). Because of its high death-to-case ratio, persistence in water supplies and its ability to occur in explosive epidemic form, cholera is a public health concern. Furthermore, because of the potential threat of weaponized *V. cholerae* to the food and water supply, it is a priority organism in biodefense research. The threat to the economy, environment and human health is also highlighted by the finding that *V. cholerae* has the potential to be transported internationally and invade new regions through the ballast water of ships (McCarthy et al., 1994). *V. cholerae* is known to persist in the environment, however, the factors

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promoting the environmental persistence of *V. cholerae* are not well understood.

[0003] *V. cholerae* can alter its phenotype and reversibly switch from EPSoff (smooth colony morphology) to EPSon (rugose colony morphology) in which the cells are embedded in extracellular polysaccharide or rugose exopolysaccharide (rEPS) and display a wrinkled "rugose" colony morphology (Figs. 1A and 1B) and an associated biofilm (White, 1940 and Rice et al., 1993). The switch to EPSon and the rugose phenotype promotes biofilm formation (Rice et al., 1993; Morris et al., 1996; and Watnick et al., 1999). Importantly, EPS is essential for *V. cholerae* biofilm formation. The rugose variant is highly chlorine resistant and shows increased resistance to killing by acid, UV light and complement-mediated serum bactericidal activity (Rice et al., 1993; Morris et al., 1996; and Yildiz et al., 1999). Therefore, switching to EPSon and the rugose phenotype might be important in niche specialization and in promoting survival and fitness in particular environments. Rugose strains are virulent and cause fluid accumulation in rabbit ileal loops, produce diarrhea in human volunteers and are highly resistant to complement-mediated bactericidal activity (Rice et al., 1993; Morris et al., 1996; and Yildiz et al., 1999). The rugose or wrinkled colony phenotype consisting of aggregating cells has been reported in *S. enterica* Enteritidis (Petter, 1993), *S. enterica* Typhimunium (Anriany et al., 2001), *V. parahaemolyticus* (Güvener et al., 2003), *P. aeruginosa* (Parsek, 2003), and *Enterobacter sakazakii* (Farmer et al., 1980). Research by the laboratory of the present inventor and others has also shown that production of *V. cholerae* EPS is linked to the type II general extracellular protein secretion pathway which is also involved in secretion of important virulence factors (Ali et al., 2000; Davis et al., 2000).

[0004] The *vps* (*Vibrio* polysaccharide) gene cluster in *V.*
cholerae carries the structural genes for the biosynthesis of
rEPS (Yildiz et al., 1999). The *vps* gene cluster is thought to
be comprised of two closely located but separate operons in which
vpsA and *vpsL* represent the first genes of each operon (Yildiz et
al., 1999 and 2001). Transcription of *vpsA* and *vpsL* is regulated
by VpsR (a homolog of σ54 transcriptional activators) by a
mechanism that is not well understood (Yildiz et al., 2001).
VpsR has high homology to NtrC, AlgB and HydG bacterial enhancer-
binding protein that activates transcription after
phosphorylation of its receiver domain by an associated sensor
kinase protein (Kern et al., 1999). Previous studies have found
that HapR in some *V. cholerae* strains is linked to the rugose
phenotype by some unknown mechanism (Jobling et al., 1997) and
CytR can repress transcription of *vps* genes and the associated
biofilm formation (Haugo et al., 2002). The present inventor has
also found that switching to the rugose phenotype in *V. cholerae*
is independent of ToxT, LuxS and RpoS (Ali et al., 2002).
However, the molecular basis underlying switch from the smooth to
the rugose phenotype of *V. cholerae* is still not well understood.

[0005] Early studies on the rugose phenotype of *V. cholerae*
were impeded by the very low frequency of switching to EPSon and
the rugose phenotype under the conditions tested (Morris et al.,
1996; Yildiz et al., 1999; and Wai et al., 1998). The laboratory
of the present inventor recently identified conditions that
promote the rapid shift (up to ~80%) to the rugose phenotype in a
process called high frequency rugose production (HFRP) (Ali et
al., 2002). It was found that there are differences in the
expression and stability of the phenotype between epidemic
strains and that the ability to switch at high frequency was more
common in epidemic *V. cholerae* strains than in nonpathogenic
strains (Ali et al., 2002). This suggests that the ability to

switch to the rugose phenotype is important in *V. cholerae* and might provide an adaptive advantage under specific conditions.

[0006] Biofilms are the primary mode of existence of many bacterial species and are central to their survival, persistence and often virulence (Costerton et al., 1995; Davey et al., 2000; Donlan, 2002 and Watnick et al., 2000). Biofilms resist environmental stresses and adverse conditions better than free-living cells, have increased nutrient availability and can better avoid immune responses (Anwar et al., 1992). A common feature of biofilms is that microorganisms are embedded in an extracellular matrix comprised mostly of EPS (Costerton et al., 1981 and Wingender et al., 1999). EPS is important for the structural and functional integrity of biofilms and determines its physicochemical and biological properties and has a role in adhesion, protection and facilitates community interactions (Wimpenny, 2000). EPS provides protection from a variety of environmental stresses such as UV radiation, pH shifts, osmotic shock, and desiccation.

[0007] The role of biofilms in the environmental persistence and transmission of certain pathogens is also well recognized. Like *V. cholerae* (Ali et al., 2002; Morris et al., 1996 and Yildiz et al., 1999), *Salmonella enterica* Typhimurium has the ability to form a rugose EPS-producing phenotype which has . increased biofilm forming ability and is proposed to have a role in increased persistence in the environment (Anriany et al., 2001). *Salmonella enteritidis* biofilms resistant to cleaning fluids have been shown to persist for at least 4 weeks in domestic toilets after episodes of salmonellosis (Barker et al., 2000). The finding that *E. coli* and *Salmonella* biofilms can be found on sprouts may make their eradication with antimicrobial compounds difficult and therefore increasing their persistence, resulting in ingestion and infection (Fett, 2000). The

importance of biofilms is also highlighted in the process of
horizontal gene transfer since some results suggest that DNA
exchange may be increased in bacteria that are attached to a
surface and in biofilms rather than between free-swimming
planktonic cells (Ehlers, 2000). This has implications in the
transfer of genes encoding functions such as antibiotic
resistance or virulence and overall persistence.

[0008] Clinically, biofilm formation is known to be a key
factor in the establishment and persistence of several difficult
to treat infections. Cystic fibrosis is caused by certain *P.
aeruginosa* strains which express copious amounts of EPS and form
biofilms in the lung (Davies et al., 1995; Geesey et al., 1993
and Govan et al., 1996). The EPS of these *P. aeruginosa* strains
makes them recalcitrant to antimicrobial treatment.
Interestingly, like the EPS of *V. cholerae* (Ali et al., 2002 and
Morris et al., 1996), alginate EPS production by *P. aeruginosa*
protects these strains against chlorine and may contribute to
survival of these bacteria in chlorinated water systems (Grobe et
al., 2001). Another example of a biofilm-mediated infection is
chronic ear infection (otitis media) (Dingman et al., 1998).
Peridontitis is also another example of a biofilm-mediated
disease that results from chronic inflammation of the tissue
supporting the gums and can lead to tooth loss. The main microbe
causing this disease is *Porphyromonas gingivalis* (Lamont et al.,
1998).

[0009] The EPS matrix of biofilms has the potential to
physically prevent access of certain antimicrobial agents into
the biofilm by acting as an ion exchanger, thereby restricting
diffusion of compounds from the external milieu into the biofilm
(Goodell et al., 1985; Nichols et al., 1988 and Nickel et al.,
1985). *Helicobacter pylori* produces a biofilm that appears to be
important in enhancing resistance to host defense factors and

antibiotics and in promoting growth under low pH conditions *in* *vivo* (Stark et al., 1999). Biofilm bacteria can be up to 1,000-fold more resistant to antibiotic treatment than the same organism grown planktonically (Gilbert et al., 1997). Clinical biofilm infections are marked by symptoms that typically recur even after repeated treatments with antibiotics. Moreover, biofilm infections are rarely resolved by the host's immune system (Costerton et al., 1999). Bacterial biofilms on prosthetic valves are the leading cause of endocarditis in patients who have undergone heart valve replacement. Among patients who develop these infections, the mortality rate is as high as 70% (Hyde et al., 1998). Millions of catheters (e.g., central line, intravenous, and urinary catheters) are inserted into patients every year, and these implants serve as a potential surface for biofilms. Overall, it is thought that upwards of 60% of all nosocomial infections are due to biofilms. These biofilm-based infections can increase hospital stays by up to 2-3 days and cost upwards of $1 billion per year in added costs (Archibald et al., 1997).

[0010] *Staphylococcus aureus* is another biofilm-forming bacteria that has long been recognized as an important human and animal pathogen (Archer, 1998; Hermans et al., 2003; Kluytmans et al., 1997 and Sutra et al., 1994). *S. aureus* can be found on the skin and mucosal surfaces of humans, particularly the anterior nares. If followed over time, ~ 20% of the human population are persistent carriers; ~60%, intermittent carriers while ~20% of the population will never be colonized (Peacock et al., 2001). *S. aureus* is a common cause of both community-acquired and hospital-acquired infections. In a recent population-based active surveillance study from Canada, the annual incidence of invasive *S. aureus* infection was 28.4 per 100,000 population (Laupland et al., 2003). Certain populations including patients

with indwelling medical devices such as vascular catheters,
patients on hemodialysis, patients who use intravenous drugs,
patients with dermatologic disease and diabetes mellitus have
higher rates of colonization than the general population (Kirmani
et al., 1978; Tuazon et al., 1975 and 1974). The *S. aureus*
carrier state is clinically important because a carrier is at
risk for infection with the colonizing strain. Studies in
patients on dialysis, patients with HIV infection and patients
with bloodstream infection support the hypothesis that *S. aureus*
isolates causing infection are endogenous in origin when strains
are examined by molecular typing (Ena et al., 1994; Luzar et al.,
1990; Nguyen et al., 1999; von Eiff et al., 2001 and Yu et al.,
1986). Hence, ways to inhibit or reduce *S. aureus* carriage and
colonization are needed.

[0011] According to the Center for Disease Control and
Prevention's National Nosocomial Infection Surveillance system,
S. aureus is particularly a common cause of nosocomial infections
and is the most common cause of surgical site infection and the
second most common cause of nosocomial bacteremia (National
Nosocomial Infections Surveillance (NNIS) Report, 1998). The
overall number of *S. aureus* infections in intensive care units
increased from 1987 to 1997 with the majority of the increase due
to *S. aureus* isolates resistant to methicillin (Lowry, 1998). *S.
aureus* is often resistant to multiple antibiotics. Infections
caused by methicillin- and multiple antibiotic resistant *S.
aureus* (MRSA) are particularly difficult to treat and MRSA
infections are often associated with higher mortality and
increased healthcare costs than methicillin-sensitive strains
(Cosgrove et al., 2003).

[0012] *S. aureus* is also a common cause of intramammary
infections (IMI) in lactating females and often results in
chronic mastitis with annual losses in the dairy industry

associated with subclinical mastitis in dairy cows across the
U.S. being estimated at approximately $1 billion (Ott, 1999).
The drug of choice for infections due to methicillin-resistant *S.
aureus* (MRSA) is vancomycin, although this antibiotic is given as
a last line of treatment.

[0013] Like other bacterial species, biofilm formation is
known to be a key factor in the establishment and persistence of
staphylococcal infections. Bacterial cells in biofilms can be up
to 1,000-fold more resistant to antibiotic treatment than the
same cells grown planktonically. Consistent with this
observation, biofilm formation on tissues or on medical devices
is an important first step in the pathogenesis of *S. aureus*
infection of humans and animals (Bradley et al., 1991; Cole et
al., 2001; Cucarella et al., 2001, 2002 and 2004; Götz, 2002;
Huang et al., 2003; Kluytmans et al., 1997; Mest et al., 1994;
Muder et al., 1991; Peacock et al., 2001; Pujol et al., 1996; and
Roghmann et al., 2001). Overall, it is thought that upwards of
60% of all nosocomial infections involve biofilms. These
biofilm-based infections can increase hospital stays by up to 2-3
days and cost upwards of $1 billion per year in added costs.
Although the risk of infection is high in people colonized with
S. aureus, there is another compelling reason to prevent
colonization and biofilm formation, which is to prevent the
transmission of *S. aureus* to others (Muto et al., 2003). MRSA
does not spontaneously emerge from existing methicillin-
susceptible *S. aureus*. The majority of people with MRSA
colonization acquire MRSA through exposure to the hands of
healthcare workers transiently colonized with MRSA from prior
contact with an MRSA infected or colonized patient (Muto et al.,
2003). Infection control measures such as isolation and
handwashing reduce but do not eliminate this transmission,
although compliance with these policies is often low (Richet et

al., 2003). Decolonization regimens, as an approach to
controlling transmission to others, have generally been
unsuccessful as the eradication of MRSA is generally only
temporary. Therefore, the development of novel intervention
strategies that prevent or inhibit colonization and biofilm
formation are needed.

[0014] Cyclic nucleotides, such as cAMP and cGMP, are well
recognized as important low-molecular weight signaling molecules
in eukaryotes. In bacteria, while cAMP has a role in alleviating
glucose catabolite repression (Jackson et al., 2002; Notley-
McRobb et al., 1997), cGMP has not been shown to act as a
signaling molecule. However, another guanosine nucleotide, the
cyclic dinucleotide c-di-GMP (also known as 3',5'-cyclic
diguanylic acid, cyclic diguanylate, cyclic diguanosine
monophosphate, cyclic bis (3'→5') diguanylic acid, cyclic
diguanylic acid, cGpGp, and c-GpGp)
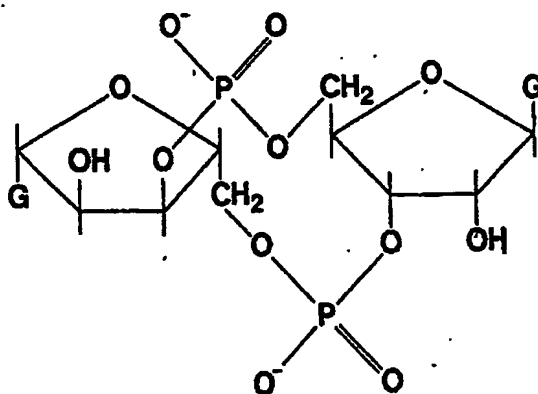
where G in the above structure is guanine, has been reported to
be an intracellular bacterial signaling molecule in a few species
and whose structure is known and consists of two cGMP molecules
bound head-to-tail (Jenal, 2004 and Ross et al., 1991). c-di-GMP
was first identified in *Acetobacter xylinum* (renamed
Gluconacetobacter xylinum) and shown to regulate cellulose

production in this species (Amikam et al., 1989; Mayer et al., 1991; Ross et al., 1990 and 1991). The exact molecular mechanism remains unclear but regulation in *G. xylinum* appears to involve c-di-GMP binding to a membrane protein that activates gene expression. Cellulose production appears to be modulated by the opposing effects of two proteins with GGDEF domains, diguanylate cyclase (Dgc) and c-di-GMP phosphodiesterase (PdeA), each controlling the level of c-di-GMP in the cell. Thus, c-di-GMP is thought to be a signaling molecule.

[0015] Based on studies by the laboratory of the present inventor and others, it is now becoming increasingly reported that biofilm formation in many pathogens including *Vibrio cholerae, Yersinia pestis, Salmonella enteritidis* Typhimurium and *Pseudomonas aeruginosa* is associated with GGDEF proteins (Bomchil et al., 2003; D'Argenio et al., 2002; Jones et al., 1999 and Römling et al., 2000).

[0016] The increasing emergence of antimicrobial resistance in bacterial pathogens and the importance of colonization and biofilm in the infection process requires that alternate antimicrobial strategies be developed. Until the present invention, the application of cyclic dinucleotides such as c-di-GMP for use as an antimicrobial approach in the control of biofilms and potentially infection has not been described.

[0017] Citation of any document herein is not intended as an admission that such document is pertinent prior art, or considered material to the patentability of any claim of the present application. Any statement as to content or a date of any document is based on the information available to applicant at the time of filing and does not constitute an admission as to the correctness of such a statement.

SUMMARY OF THE INVENTION

[0018] The present invention provides a method for attenuating the virulence of a microbial pathogen or for inhibiting or reducing colonization by a microbial pathogen, regardless of whether or not the microbial pathogen is a biofilm-forming bacteria, by administering c-di-GMP or a cyclic dinucleotide analogue of c-di-GMP to a patient in need thereof. This method is capable of treating bacterial infections.

[0019] For biofilm-forming bacteria, the present method also inhibits biofilm formation or reduces its presence, i.e., the amount of pre-formed biofilm. Thus, the present invention provides a method for inhibiting biofilm formation or for reducing the amount of pre-formed biofilm and inhibiting further biofilm development to thereby treat an infection caused by a biofilm-forming bacterial pathogen.

[0020] The present invention is also directed to a method for inhibiting microbial colonization and biofilm formation and for reducing colonization and promoting biofilm dissolution (reducing pre-existing colonization and pre-formed or accumulated biofilm) on a solid surface, in particular, a solid surface of a medical device that is or comes into close contact with a patient, by exposing the solid surface with c-di-GMP or a cyclic dinucleotide analogue thereof.

[0021] A further aspect of the present invention is directed to a pharmaceutical composition containing c-di-GMP or a cyclic dinucleotide thereof as an active ingredient.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] Figures 1A and 1B show colony morphology of the smooth (Fig. 1A) and rugose (Fig. 1B) variants of *V. cholerae* strain N16961. Colonies are shown following 48 h on LB agar plates.

[0023] Figure 2 is a graph showing biofilm formation by smooth and rugose colony variants of *V.cholerae*.

[0024] Figure 3 is a Swarm plate assay showing the motility of *V. cholerae* strains N16961 (wildtype), AmiB mutant (DK630) and RocS mutant (DS567). Plates contain LB media supplemented with 0.3% agar and were incubated at 37°C for 4 h.

[0025] Figures 4A and 4B show the effect of an AmiB mutation on the cellular morphology of *V. cholerae* strain N16961, where wildtype cells are shown in Fig 4A and AmiB mutant cells are shown in Fig. 4B. Note that the AmiB cells have a difference in morphology and show an increase in overall cell size and the presence of numerous cells in chains. Magnification 1000x.

[0026] Figures 5A and 5B show the HPLC profile of c-di-GMP. Analysis immediately after synthesis of c-di-GMP showing the purity of the product (Fig. 5A). Analysis showing the purity of c-di-GMP in 0.9% NaCl after 3 months storage at 4°C (Fig. 5B).

[0027] Figures 6A-6F show the effect of c-di-GMP on *S. aureus* cell-to-cell aggregation. 24 h culture of DK825 treated with 200 μM c-di-GMP (Fig. 6A) and untreated control (Fig. 6B). Gram stain of c-di-GMP treated cells (Fig. 6C) and untreated cells (Fig. 6D). c-di-GMP treated culture of *bap* mutant M556 (Fig. 6E) and untreated control (Fig. 6F). Magnification in Figs. 6C and 6D is 630X.

[0028] Figures 7A-7C show the effect of c-di-GMP on the ability of *S. aureus* human clinical isolates to form biofilms on a polystyrene surface using microtiter plates. Inhibition of biofilm formation in wells of a microtiter plate by *S. aureus* strain DK825 in TSB and 0.25% glucose treated with various concentrations of c-di-GMP for 24 h and stained with crystal violet. Visual appearance and O.D.$_{570}$ values of the wells are shown (Fig. 7A). Quantitative analysis of dose-response of the inhibition of biofilm formation in *S. aureus* strain DK825 treated

with c-di-GMP (Fig. 7B). Quantitative analysis of the inhibition
of biofilm formation in hyperbiofilm *S. aureus* strain 15981
treated with c-di-GMP (Fig. 7C).

[0029] Figures 8A-8D are graphs showing quantitative analysis
on the effect of c-di-GMP on the ability of *S. aureus* bovine
mastitis isolates V329 (Fig. 8A), M556 (Fig. 8B), V299 (Fig. 8C),
and V315 (Fig. 8D) to form biofilms on a polystyrene surface.

[0030] Figures 9A and 9B show the effect of guanosine
nucleotide analogs on inhibiting *S. aureus* biofilm formation on
polystyrene surfaces. Inhibition of biofilm formation in wells
of a microtiter plate by *S. aureus* DK825 in TSB and 0.25% glucose
treated with the nucleotides c-di-GMP, cGMP and 5'-GMP. Visual
appearance and O.D.$_{570}$ values of the wells are shown in Fig. 9A.
Quantitative biofilm analysis on the effect of 5'-GMP, cGMP and
c-di-GMP treatment on biofilm formation is shown in Fig. 9B.

[0031] Figure 10 is a graph showing quantitative biofilm
analysis on the effect of c-di-GMP on *S. aureus* 24 h pre-formed
biofilms.

[0032] Figures 11A and 11B show the effect of c-di-GMP
treatment on *S. aureus* DK825 adherence to HeLa epithelial cells.
Fig. 11A, untreated control culture; Fig. 11B, c-di-GMP treated
culture.

[0033] Figure 12 is a graph showing the effect of c-di-GMP on
biofilm formation in *V. cholerae* strain N16961 and in a RocS
mutant. Results are averages based on at least three independent
colonies.

[0034] Figure 13 is a schematic representation of a biofilm
growth reactor system, which is a once-through system entirely
enclosed within a 37°C incubator. 10^7 CFUs are injected into the
reactor tubing and allowed to attach for 30 min at which time
flow is restored to the system. Biofilms may be harvested by

removing the flow-cell or opening the silicon tubing and scraping the inner lumen.

[0035] Figure 14 shows a sealed flowcell (Protofab, Inc., Bozeman, MT) which is inserted inline into the biofilm growth reactor system shown in Figure 13 and is used to obtain biofilm samples attached to various surfaces including PMMA and stainless steel. Inserts are easily removed for biofilm harvest.

[0036] Figure 15 is a graph showing the effect of c-di-GMP on CFU counts of *S. aureus* Newbould 305 strain in a mouse mastitis model.

DETAILED DESCRIPTION OF THE INVENTION

[0037] The present inventor has discovered that the cyclic dinucleotide, c-di-GMP (3',5'-cyclic diguanylic acid, c-GpGp) is a naturally occurring signal (effector) molecule that affects microbial biofilm formation and plays a prominent role in colonization, motility and virulence of pathogenic bacteria. Pure chemically synthesized c-di-GMP is soluble and stable, and treatment of *S. aureus* with c-di-GMP demonstrates dramatically reduced biofilm formation and cell-to-surface interactions of *S. aureus*, and a striking anti-clumping effect on *S. aureus* cells inhibiting cell-to-cell bacterial interactions. Results obtained by the present inventor further demonstrate that c-di-GMP greatly inhibits the adherence of *S. aureus* to human epithelial cells, shows no significant toxicity in several cell lines, and was non-lethal in mice at biologically relevant doses. Thus, c-di-GMP inhibits biofilm formation in *S. aureus* and reduces or attenuates its virulence and its ability to colonize.

[0038] Further experiments have found that c-di-GMP affects the expression of numerous genes in *S. aureus*. For instance, quorum sensing genes were up-regulated and genes associated with toxin production, virulence, adhesion and colonization were down-

regulated. These results are consistent with the rôle of quorum
sensing genes as regulators with known roles in virulence, toxin,
colonization and biofilm-associated genes, and further support
the finding that c-di-GMP attenuates biofilm formation,
colonization, cell clumping, toxin activity and virulence.

[0039] Bacterial cells have the ability to control expression
of specific genes by secreting low molecular weight signaling
molecules in association with the growth phase in a process
called quorum sensing. Physiological processes controlled by
quorum sensing occur in diverse species of bacteria and include
bioluminescence, antibiotic synthesis, pathogenicity or
virulence, protein secretion, capsular exopolysaccharide
synthesis, biofilm formation and motility (Miller et al., 2001;
Schander et al., 2001; Whitehead et al., 2001). In *V. cholerae*,
biofilm formation and several other phenotypes important for
virulence are known to be regulated by small signaling molecules
in quorum sensing (Hammer et al., 2003; Miller et al., 2002; Zhu
et al., 2002). However, c-di-GMP has not been previously
identified as such a signaling molecule for quorum sensing.
Controlling cell density and virulence is one of the prominent
features of quorum sensing and compounds that cause defects in
quorum sensing would have antibacterial activity. Accordingly,
one aspect of the present invention provides a method for using
c-di-GMP or cyclic dinucleotide analogues thereof to disrupt or
inhibit quorum sensing communication regulatory systems in
pathogenic bacteria.

[0040] From the results with *S. aureus*, it is expected that c-
di-GMP is a universal signaling molecule in bacteria (regardless
of whether or not the bacteria is a biofilm-forming bacteria) and
therefore is also involved in such physiological processes as
biofilm formation, toxin production, colonization and virulence
in pathogenic bacteria. The present invention provides a method

for attenuating the virulence of a microbial pathogen or for
inhibiting or reducing colonization by a microbial pathogen which
involves administering to a patient in need thereof, i.e., a
patient exposed to, colonized by, or infected with a microbial
pathogen, an effective amount of c-di-GMP or a cyclic
dinucleotide analogue of c-di-GMP. Thus, by attenuating the
virulence of the microbial pathogen, the present method is able
to treat bacterial infections, either by using c-di-GMP (or a
cyclic dinucleotide thereof) alone or synergistically in
combination with another antibiotic/antimicrobial agent. For
instance, the inhibition of biofilm formation would certainly
make a pathogenic bacteria much more susceptible to the action of
another antibiotic/antimicrobial agent, such as those
conventionally used to treat pathogen-specific infections. The
terms "treatment", treating" and "to treat" are intended to not
only be directed to active or established bacterial infections
but also to inhibiting the initial stages of pathogenesis leading
to infection.

[0041] As a preferred embodiment, the present method inhibits
biofilm formation in a microbial pathogen for which the formation
of a biofilm is critical to its pathogenicity, i.e., its
virulence and its ability to colonize. For *S. aureus*, c-di-GMP
is administered to a patient in need thereof to inhibit *S. aureus*
colonization and biofilm formation (or reduce the colonization
and biofilm already formed) and to treat an *S. aureus* infection.
S. aureus is known to cause a wide variety of human and animal
infections including, but not limited to, impetigo, mastitis,
food poisoning, sepsis, osteomyelitis, arthritis, endocarditis,
and pneumonia. Preliminary data in an animal (mastitis) model of
infection show that c-di-GMP inhibits mastitis, an *S. aureus*
infection of the mammary gland. Mastitis in dairy cows (bovine

mastitis) is of particular concern and economic importance in the dairy industry.

[0042] Using *Staphylococcus aureus* as an example, the presence of *S. aureus* in a hospital environmental poses a risk of colonization or infection in hospital patients and personnel. Accordingly, c-di-GMP can be administered to hospital patients and personnel and new incoming patients, i.e., by spraying skin, nasal and mucosal surfaces, to inhibit *S. aureus* colonization and biofilm formation on patients and to reduce the colonization and biofilm formation of those individuals who are carriers for *S. aureus*.
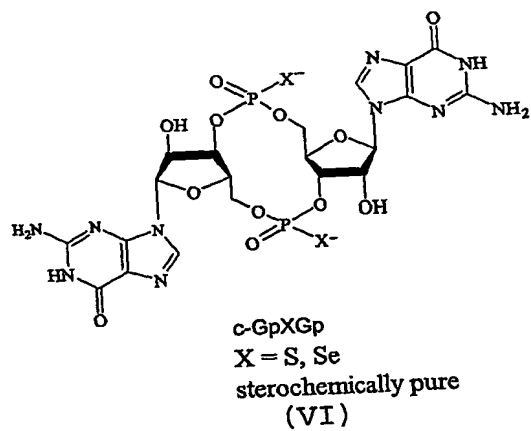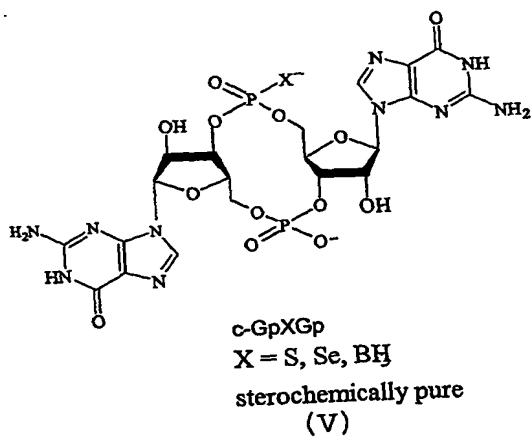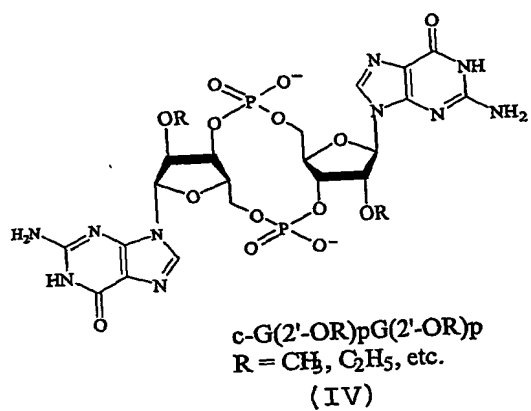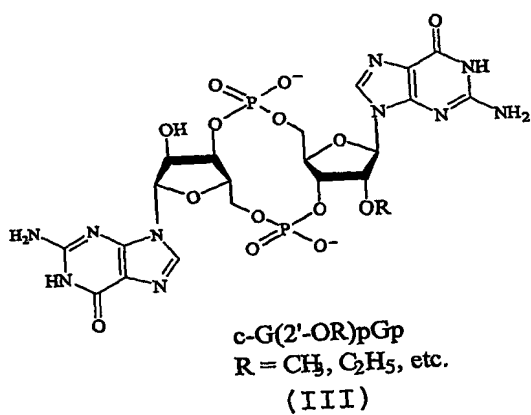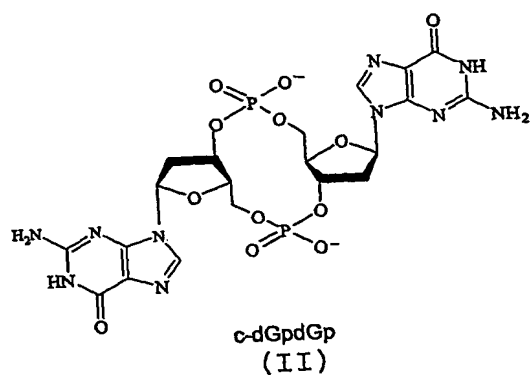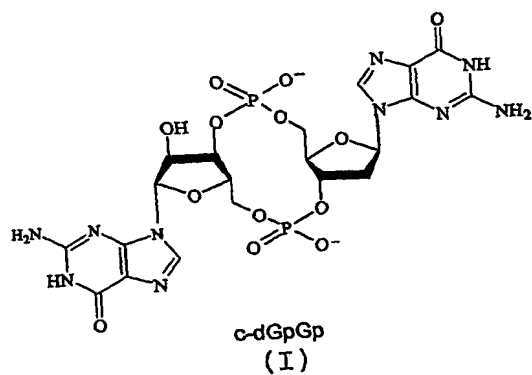
[0043] Besides c-di-GMP, a cyclic dinucleotide analogue thereof which acts as a c-di-GMP agonist, i.e., having the same effect as c-di-GMP, can be used to inhibit *S. aureus* colonization and biofilm formation (or reduce pre-existing colonization and pre-formed biofilm) and to treat *S. aureus* infection.

[0044] The present inventor has further surprisingly found that, depending on the bacteria, c-di-GMP may have the effect of inhibiting biofilm formation or it may have the opposite effect of inducing or enhancing biofilm formation. For instance, in *Vibrio cholerae* and *Salmonela enteritidis*, both of which are gram-negative, c-di-GMP was found to induce or enhance biofilm formation, the opposite of its effect in *S. aureus*, a gram-positive bacteria. Thus, the effect of c-di-GMP is bacteria-specific. Nevertheless, regardless of whether c-di-GMP inhibits or whether it enhances biofilm formation, c-di-GMP still functions as a signaling effector molecule that modulates biofilm formation in bacteria.
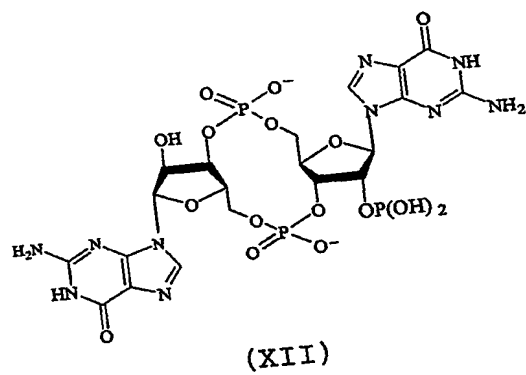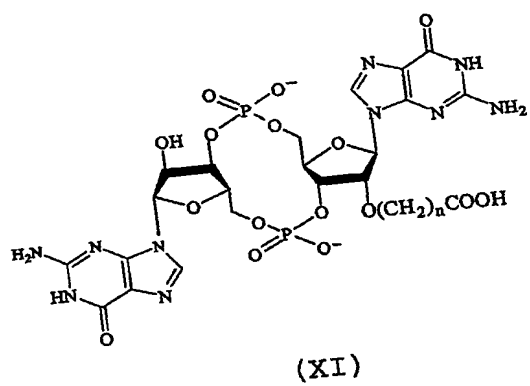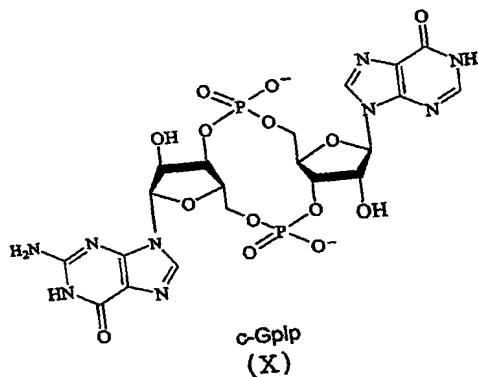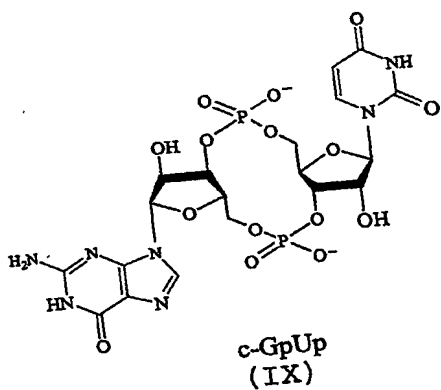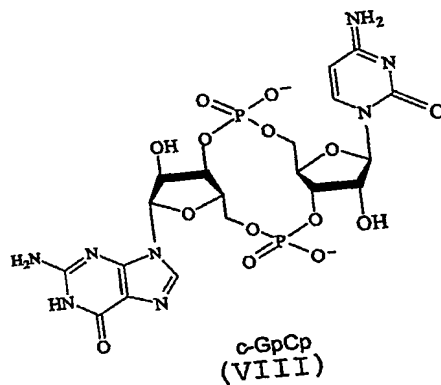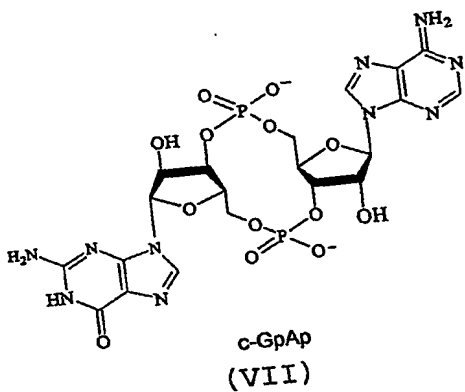
[0045] While it is possible that the phenomenon of opposite effects of c-di-GMP in different bacteria may be due to a bacteria being gram positive versus being gram negative, this is mere speculation, which can be easily tested. The biofilm
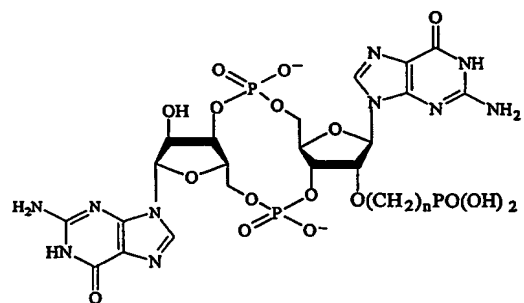
formation/inhibition assays in microtiter plates or in test tubes
and flasks, as disclosed in the examples hereinbelow, are quick
and easy assays for determining the effect of c-di-GMP on a
particular bacteria. Moreover, these assays can just as easily
accommodate the testing of many different types of bacteria,
i.e., many different strains, species and/or genera, for the
effect of c-di-GMP with high throughput. Thus, for any biofilm-
forming bacteria, the bacteria can be easily and rapidly tested
for the effect of c-di-GMP on its biofilm formation. If c-di-GMP
is found to inhibit biofilm formation, then c-di-GMP or a cyclic
dinucleotide analogue having c-di-GMP activity (acts as a c-di-
GMP agonist) can be used to inhibit biofilm formation or to
reduce pre-formed biofilm (as well as to attenuate virulence and
to inhibit and reduce colonization). Likewise, if c-di-GMP
instead enhances or induces biofilm formation, then a cyclic
dinucleotide analogue of c-di-GMP having c-di-GMP antagonist
activity (acts opposite to the effect of c-di-GMP) can be used to
inhibit biofilm formation or to reduce pre-formed biofilm (as
well as to attenuate virulence and to inhibit and reduce
colonization). *V. cholerae* and *S. enteritidis* are non-limiting
examples of bacteria in which an added cyclic dinucleotide
analogue of c-di-GMP that acts as a c-di-GMP antagonist inhibits
biofilm formation.

[0046] It will be appreciated by those of skill in the art
that not only can the quick and easy biofilm formation/inhibition
assays be used to rapidly determine the effect of c-di-GMP on the
bacteria tested, but the assays can also be used to determine
with only routine experimentation if a cyclic dinucleotide
analogue of c-di-GMP is an agonist or antagonist of c-di-GMP.
Non-limiting examples of cyclic dinucleotide analogues of c-di-
GMP are presented below as compounds (I)-(XIX):

c-dGpGp
(I)

c-dGpdGp
(II)

c-G(2'-OR)pGp
R = CH₃, C₂H₅, etc.
(III)

c-G(2'-OR)pG(2'-OR)p
R = CH₃, C₂H₅, etc.
(IV)

c-GpXGp
X = S, Se, BH₃
sterochemically pure
(V)

c-GpXGp
X = S, Se
sterochemically pure
(VI)

c-GpAp
(VII)

c-GpCp
(VIII)

c-GpUp
(IX)

c-GpIp
(X)

(XI)

(XII)

(XIII)
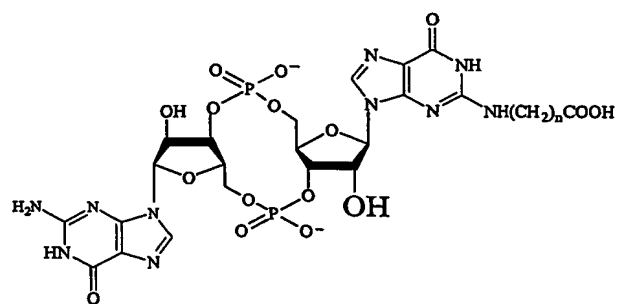
(XIV)
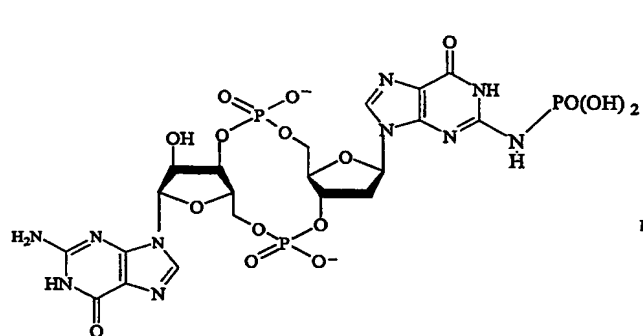
(XV)

(XVI)

(XVII)

(XVIII)

(XIX)
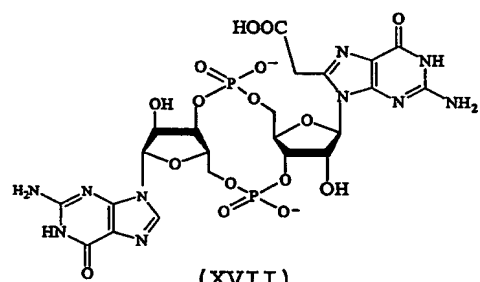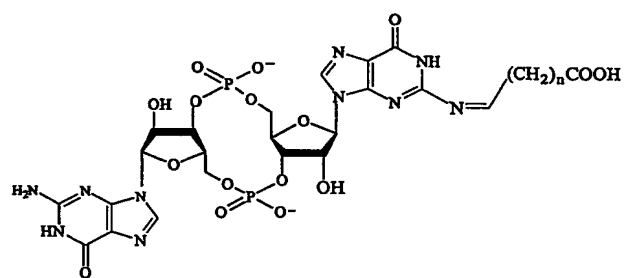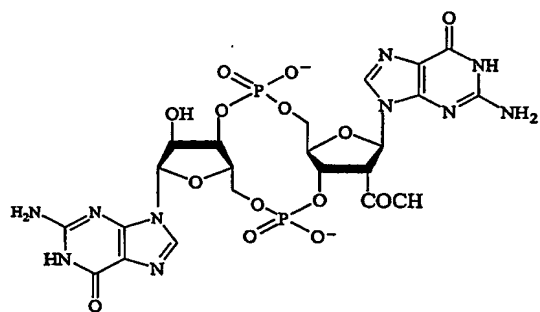
The above cyclic dinucleotides are only preferred embodiments of
the cyclic dinucleotide analogues of c-di-GMP and are not
intended to be limiting. For example, the guanine base can be
substituted with other bases.

[0047] The present invention also provides a method for
inhibiting microbial colonization and biofilm formation on a
solid surface by exposing the solid surface to an effective
amount of c-di-GMP or a cyclic dinucleotide analogue thereof to
inhibit microbial colonization and biofilm formation or reduce
its presence on the surface of a medical device, more preferably
a medical device which is implantable or implanted in a patient,
i.e., artifical joints, stents, etc., or is otherwise attached or
in close contact with the patient, i.e., catheters, in-dwelling
devices, intravenous tubing system, insulin pump, etc. It will
also be appreciated that the solid surface can be on non-
medically oriented devices, such as an industrial pipeline, and
buildings or construction material where the presence of biofilm-
forming bacteria can cause problems that require remediation. As
the microbial biofilm formed by S. aureus is of particular
concern on the solid surfaces of medical devices, a preferred
embodiment of this aspect of the invention is to inhibit S.
aureus biofilm formation or reduce its presence on a solid
surface of a medical device by exposing the surface to an
effective amount of c-di-GMP agonist. In this method, as in the
method discussed above for attenuating the virulence of a
microbial pathogen and for inhibiting colonization and biofilm
formation, the c-di-GMP or a cyclic dinucleotide analogue thereof
(either agonist or antagonist) can be selected based on the type
of biofilm forming bacteria that is of concern for a particular
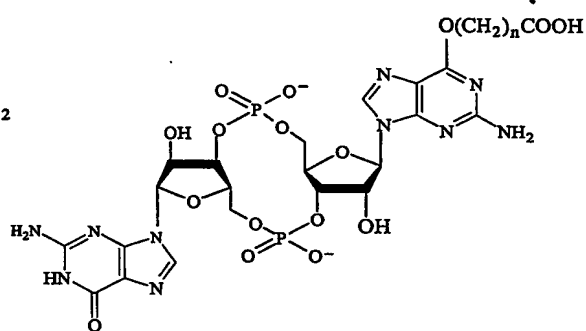surface. For instance, c-di-GMP can be used if S. aureus or a
bacteria whose biofilm formation is inhibited by c-di-GMP is of
main concern. In other instances, a cyclic dinucleotide analogue

of c-di-GMP which acts as a c-di-GMP antagonist can be used when
the bacterial biofilm formation of concern is enhanced/induced by
c-di-GMP but is inhibited by an antagonist of c-di-GMP.

[0048] Those of skill in the art will appreciate that the
solid surface can be exposed to c-di-GMP or a cyclic dinucleotide
analogue thereof in any number of ways known to those of skill in
the art. One way could be to attach or immobilize c-di-GMP or a
cyclic dinucleotide analogue thereof on the surface or to
incorporate c-di-GMP or a cyclic dinucleotide analogue thereof in
the surface. Another way could be to flush the solid surface
with a solution containing c-di-GMP or a cyclic dinucleotide
analogue thereof.

[0049] Non-limiting examples of the variety of bacterial
species, both pathogenic and non-pathogenic, for which the
methods of the present invention are appropriate include *Vibrio
harveyi, Vibrio cholerae, Vibrio parahaemolyticus, Vibrio
alginolyticus, Pseudomonas fluorescens, Pseudomonas aeruginosa,
Pseudomonas acidovorans, Pseudomonas alcaligenes, Pseudomonas
putida, Pseudomonas syringae, Pseudomonas aureofaciens,
Pseudomonas fragi, Fusobacterium nucleatum, Treponema denticola,
Citrobacter freundii, Porphyromonas gingivalis, Moraxella
catarrhalis, Stenotrophomonas maltophilia, Burkholderia cepacia,
Aeromonas hydrophilia, Salmonella typhi, Salmonella paratyphi,
Salmonella Enteritidis, Shigella dysenteriae, Shigella flexneri,
Shigella sonnei, Enterobacter cloacae, Enterobacter aerogenes
Yersinia enterocolitica, Yersinia pestis, Yersinia
pseudotuberculosis, Yersinia inten-nedia, Bordetella pertussis,
Bordetella parapertussis, Bordetella bronchiseptica, Escherichia
coli, Salmonella typhimurium, Haemophilus influenzae,
Haemophilus, parainfluenzae, Haemophilus haemolyticus,
Haemophilus parahaemolyticus, Pasteurella multocida, Pasteurella
haemolytica, Gardnerella vaginalis, Bacteroides spp., Clostridium*

difficile, *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycrobacterium. leprae*, *Corynebacterium diplitheriae*, *Corynebacterium ulcerans*, *Legionella pneurnophila*, *Listeria monocytogenes Helicobacter pylori*, *Bacillus subtilis*, *Bacillus anthracis*, *Borrelia burgfdorferi*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Borrelia burgdorferi*, *Campylobacter fetus*, *Campylobacterjejuni*, *Campylobacter coli,*, *Deinococcus radiodurans*, *Mycobacterium tuberculosis*, *Desulfvibrio spp.*, *Actinomyces spp.*, *Erwinia spp.*, *Xanthomonas spp.*, *Xylella spp.*, *Clavibacter spp.*, *Desulfomonas spp.*, *Desulfovibrio spp.*, *Desulfococcus spp.*, *Desulfobacter spp.*, *Desulfobulbus spp.*, *Desulfosarcina spp.*, *Desulfuromonas spp.*, *Acinetobacter calcoaceticus*, *Acinetobacter haemolyticus*, *Enterococcus faecalls*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Serratia marcescens*, *Francisella tularensis*, *Morganella morganii*, *Providencia alcalifaciens*, *Providencia rettgeri*, *Providencia stuartii*, *Proteus mirabilis*, *Proteus vulgarls*, *Streptomyces spp.*, *Clostridium. spp.*, *Rhodococcus spp.*, *Thermatoga spp.*, *Sphingomonas spp.*, *Zymomonas spp.*, *Micrococcus spp.*, *Azotobacter spp.*, *Norcardia spp.*, *Brevibacterium spp.*, *Alcaligenes spp.*, *Microbispora spp.*, *Micromonospora spp.*, *Methylobacterium organophilum*, *Pseudomonas reptilivora*, *Pseudomonas carragienovora*, *Pseudomonas dentificans*, *Corynebacterium spp.*, *Propionibacteriurn spp.*, *Xanothomonas spp.*, *Methylobacterium spp.*, *Chromobacteriurn spp.*, *Saccharopolyspora spp.*, *Actinobacillus spp.*, *Alteromonas spp.*, *Aeronomonas spp.*, *Agrobacterium tumefaciens*, *Staphylococcus aureus*, *Staphylococcus epidennidis*, *Staphylococcus hominis*, *Staphylococcus. haemolyticus; Staphylococcus warneri*, *Staphylococcus cohnii*, *Staphylococcus saprophyticus*, *Staphylococcus capitis*,

Staphylococcus lugdunensis, Staphylococcus intemedius,
Staphylococcus hyicus, Staphylococcus saccharolyticus and
Rhizobium. spp., and mutants thereof.

[0050] The method of the present invention for attenuating the
virulence of a microbial pathogen or for inhibiting or reducing
colonization by a microbial pathogen is intended to be used
preferably in mammals, most preferably in humans, but can also be
used in other animals such as birds.

[0051] Pharmaceutical compositions containing c-di-GMP or a
cyclic dinucleotide analogue thereof for use in accordance with
the method of the present invention for attenuating the virulence
of the microbial pathogen or for inhibiting or reducing
colonization by a microbial pathogen may be formulated in
conventional manner using one or more physiologically acceptable
carriers or excipients. The carrier(s) must be "acceptable" in
the sense of being compatible with the other ingredients of the
composition and not deleterious to the recipient thereof.

[0052] The following exemplification of carriers, modes of
administration, dosage forms, etc., are listed as known
possibilities from which the carriers, modes of administration,
dosage forms, etc., may be selected for use with the present
invention. Those of ordinary skill in the art will understand,
however, that any given formulation and mode of administration
selected should first be tested to determine that it achieves the
desired results. It will also be appreciated that c-di-GMP or a
cyclic dinucleotide thereof may be used alone as the active
ingredient or in combination with another antibiotic or anti-
microbial agent such as those conventionally used to treat
bacterial infections.

[0053] The term "carrier" refers to a diluent, adjuvant,
excipient, or vehicle with which the c-di-GMP or cyclic
dinucleotide thereof is administered. The carriers in the

pharmaceutical composition may comprise a binder, such as microcrystalline cellulose, polyvinylpyrrolidone (polyvidone or povidone), gum tragacanth, gelatin, starch, lactose or lactose monochydrate; a disintegrating agent, such as alginic acid, maize starch and the like; a lubricant or surfactant, such as magnesium stearate, or sodium lauryl sulphate; a glidant, such as colloidal silicon dioxide; a sweetening agent, such as sucrose or saccharin; and/or a flavoring agent, such as peppermint, methyl salicylate, or orange flavoring.

[0054] Methods of administration include, but are not limited to, parenteral, e.g., intravenous, intraperitoneal, intramuscular, subcutaneous, mucosal (e.g., oral, intranasal, buccal, vaginal, rectal, intraocular), intrathecal, topical and intradermal routes. Administration can be systemic or local.

[0055] For oral administration, the pharmaceutical preparation may be in liquid form, for example, solutions, syrups or suspensions, or may be presented as a drug product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinized maize starch, polyvinyl pyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica);

disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated, i.e., enterically-coated by methods well-known in the art.

[0056] Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

[0057] For topical administration, c-di-GMP or a cyclic dinucleotide analogue thereof is incorporated into topically applied vehicles such as salves or ointments.

[0058] For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

[0059] The compositions may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen free water, before use.

[0060] The compositions may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

[0061] For administration by inhalation, the compositions for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas.

A nasal spray, which does not require a pressurized pack or nebulizer as in an inhalation spray, can alternatively be used for intranasal administration. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin, for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

[0062] A typical regiment for treatment includes administration of an effective amount over a period of several days, up to and including between one week and about six months.

[0063] The effective dose at the site of colonization, biofilm formation or infection appears to be in the micromolar range, such as between about 1 μM and 990 μM, preferably about 20 μM to 500 μM, more preferably about 100 μM to 300 μM. It is within the skill of those in the pharmaceutical art to determine with routine experimentation what dosage of c-di-GMP or a cyclic dinucleotide analogue thereof will be needed, depending on route of administration, to deliver such an effective dose to the site of biofilm formation or infection.

[0064] It is understood that the dosage of c-di-GMP or a cyclic dinucleotide analogue thereof administered in vivo may be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the pharmaceutical effect desired. The ranges of effective doses provided herein are not intended to be limiting and represent preferred dose ranges. However, the most preferred dosage may be tailored to the individual subject, as is understood and determinable by one skilled in the relevant arts. See, e.g., Berkow et al., eds., The Merck Manual, 16th edition, Merck and co., Rahway, N.J., 1992; Goodman et al., eds., Goodman and Gilman's The Pharmacological Basis of Therapeutics, 8th

edition, Pergamon Press, Inc., Elmsford, N.Y. (1990); Katzung, *Basic and Clinical Pharamacology*, Appleton and Lange, Norwalk, Conn., (1992); *Avery's Drug Treatment: Principles and Practic of Clinical Pharmacology and Therapeutics*, 3rd edition, ADIS Press, LTD., Williams and Wilkins, Baltimore, MD (1987), Ebadi, *Pharmacology*, Little, Brown and Col, Boston, (1985), which references are entirely incorporated herein by reference.

[0065] Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration and are not intended to be limiting of the present invention.

EXAMPLE 1

[0066] Researchers studying the rugose phenotype of *V. cholerae* (and other species) have been impeded by the very low (<1%) frequency of switching between smooth (EPSoff) and rugose cells (EPSon) *in vitro* (Morris et al., 1996; Wai et al., 1998; White, 1940 and 1938; Yildiz et al., 1999). The laboratory of the present inventor has identified culture media and conditions, APW#3 (1% proteose peptone #3, 1% NaCl, pH 8.5.), which results in a high frequency shift of smooth cells (EPSoff) to the rugose phenotype (EPSon). This process is called high frequency rugose production (HFRP) (Table 1) (Ali et al., 2002).

TABLE 1. Frequency of switching to rugose EPS production (HFRP) by *V. cholerae* strains.

Strains[a]	Serogroup/ Biotype	Source[b]	% Rugose colonies			
			Flask		Tube	
			30°C	37°C	30°C	37°C
N16961	O1/El Tor	C (1971)	24-38	42-51	68-74	60-80
C6709	O1/ElTor	C (1991)	1	23	15	70
NCTC 6585	O1/classical	C (1943)	33-48	44-45	0	0
AMS20A73	O1/classical	C (1945)	3	4	0	0
Aldova	O37	C (1965)	0	1	71-72	23-50
1803	non-O1	C (1992)	0	0	16	87
1837	O139	C (1992)	0	0.2	0	0-2
P44	non-O1	E (2000)	12	0	0	2
1085-93	O37	E (1993)	0	0	0.1	0
141-94	O70	E (1994)	0	0	0.3	0
928-93	O6	E (1993)	0	0.2	0.4	0

[a] Listed only are strains showing HFRP or spontaneous rugose colonies.
[b] C, clinical; E, environmental; Year isolated is in parentheses.

[0067] Switching to the rugose phenotype at high frequency was found to be more common in epidemic strains than in nonpathogenic strains. It was found that 6/19 toxigenic isolates (32%) that were temporally and geographically unrelated and only 1/16 unrelated nontoxigenic strains (6%) could shift to the rugose phenotype (EPSon) and showed HFRP (T test; P<0.05) (Table 1). Of all the strains tested, El Tor strain N16961 had the highest switching rates (up to 80%). Reversion, albeit at a lower frequency, from the rugose phenotype to the smooth phenotype was also found showing that phenotypic switching is conditionally transient. These features suggest the switching process might be associated with phase variation-like mechanism. While not all epidemic strains could switch at high frequency, these results showing that switching at high frequency is more correlated with toxigenic strains suggest it is important in V. cholerae and also suggest a link between this process and virulence. Consistent with previous studies (Morris et al., 1996), a low frequency (<0.5%) shift to the rugose phenotype was found in several

strains. While it is possible that nonpathogenic strains might
have to be grown under different conditions to stimulate
switching to the EPSon rugose phenotype, this would still
nevertheless indicate that there is a difference between clinical
and nonpathogenic strains. The laboratory of the present
inventor found that a sixth pandemic (classical biotype) strain,
NCTC 6585, switched at high frequency to the rugose phenotype (up
to 48%). HFRP was defined as a >3% shift from the smooth to
rugose phenotype (Ali et al., 2002). To confirm the rugose
variant of NCTC 6585 expressed rEPS, transmission electron
microscopy (TEM) was performed on ruthenium red stained thin
sections. For TEM, 2 day old smooth and rugose colonies on LB
agar were removed as 0.5-cm^2 blocks then fixed and stained in a
solution of 2% glutaraldehyde, 0.075% ruthenium red, 50 mM lysine
monohydrochloride in 0.1 M cacodylate buffer (pH 7.2) for 1 h at
room temperature then 18 h at 4°C. Samples were washed twice in
0.1 M cacodylate buffer (pH 7.2), encased in 2% molten Noble agar
and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer
(pH 7.2) overnight at 4°C. Samples were then dehydrated in 30%,
50%, 70% and 90% EtOH for 10 min each and twice in 100% EtOH for
15 min each, followed by two treatments with propylene oxide 15
min each then infiltrated using a 1:1 solution of propylene oxide
and epon for 2 h at room temperature then in 3:1 epon/propylene
oxide overnight. Samples were then placed in pure epon for 1 h,
embedded in epon and put in a 60°C oven for 2 days then thin
sectioned (50-80 nm thick). Sections were stained with uranyl
acetate for 20 min then lead citrate for 20 min. Samples were
examined under a JEOL 1200 EX II transmission microscope at 80
kV. TEM of rugose NCTC 6585 showed the presence of extracellular
polysaccharide between cells and the absence of this material
from smooth cells. It appears that all major epidemic clones of

V. cholerae (classical, El Tor and O139) can shift to the rugose phenotype.

[0068] Production of rEPS is known to promote resistance of El Tor strains to a variety of environmental stresses such as chlorine, UV light, hydrogen peroxide, and complement-mediated bactericidal activity (Morris et al., 1996; Rice et al., 1993; Watnick et al., 1999 and Yildiz et al., 1999). In order to determine whether rugose cells of the 6th pandemic classical biotype strain NCTC 6585 promoted resistance to environmental stresses, smooth and rugose variants were exposed to chlorine. Chlorine resistance was assayed (four independent experiments) by using a 1:50 dilution of an overnight culture of NCTC 6585 in 3 ml of fresh LB (Miller) broth. Cultures were then incubated statically at 37°C for 3 h until CFU/ml ~2 x 10^8 CFU/ml, the cells harvested by centrifugation and resuspended in phosphate buffered saline (PBS) (pH 7.2) containing 3 mg/L free chlorine (sodium hypochlorite, Sigma). Following 5 min exposure to 3 mg/L chlorine, cultures were serially diluted and plated on LB agar to determine the number of surviving cells. Consistent with El Tor strain 92A1552 (Yildiz et al., 1999), rugose NCTC 6585 cells were 10,000-fold more resistant to chlorine (5 min. exposure to 3 mg/L) than smooth cells. These findings are the first to report the rugose phenotype by classical biotype strains and shows that rEPS also promotes the survival of classical biotype strains.

Switching to EPSon and the rugose phenotype promotes biofilm formation

[0069] As the rugose phenotype can promote biofilm formation in El Tor and O139 strains, the ability of smooth and rugose variants of N16961 (El Tor), NCTC6585 (classical) and Aldova (non-O1/non-O139) strains to form biofilms was tested using previously described methods (Watnick et al., 2001). Glass test

tubes containing 500 µl LB broth were inoculated with a 1:100 dilution of overnight culture of each variant.

[0070] These cultures were then incubated statically at room temperature for 24 h. Culture supernatants were then discarded, tubes rinsed vigorously with distilled water to remove non-adherent cells, filled with 600 µl 0.1% crystal violet (Sigma), incubated for 30 min at room temperature and again rinsed with water. Quantitative biofilm formation was assayed by measuring optical density at 570 nm of the solution produced by extracting cell associated dye with 600 µl DMSO (Sigma). Consistent with other studies (Yildiz et al., 1999), the results show that rugose variants of all strains tested had significantly greater (~7-fold) biofilm forming ability than smooth cells (Fig. 2) and that EPS is essential for *V. cholerae* biofilm formation.

V. cholerae can switch to the rugose phenotype (EPSon) in the environment

[0071] The supposition that switching to EPSon and the rugose phenotype promotes the survival of *V. cholerae* in the environment is based on the premise that switching to EPSon occurs in the environment. However, there have been no reports detecting rugose *V. cholerae* from environmental (or clinical) sources. Unfortunately, there is no current enrichment method for isolating rugose strains from the environment and while TCBS is a selective and differential media for *V. cholerae*, the laboratory of the present inventor has found that TCBS inhibits (masks) the rugose phenotype (Ali et al., 2002).

[0072] To test whether smooth cells switch to the rugose phenotype in natural environmental water samples, natural lake water from Lake Kittamaqundi that is located on the edge of the City of Columbia, Howard County, Maryland was used. Lake Kittamaqundi is a 27-acre man-made lake approximately 1 mile long

by 1/8th mile wide and has a maximum depth of 7 feet. The
Chesapeake Bay, which is approximately only several miles away,
is known to be a natural reservoir of *V. cholerae*. Fresh water
samples were collected from the lake between the warmer months of
March-September, 2002. At collection, the lake water had pH 7.6
and Na^+ and Cl^- concentrations of 6 mM and 2 mM, respectively.
Lake water was autoclaved for 1 h prior to use. In this study,
V. cholerae strain N16961 was grown in LB broth overnight at
37°C, centrifuged, washed twice with 0.85% NaCl, resuspended in
PBS, appropriately diluted and inoculated into 100 ml lake water
to a final concentration of 10^4-10^5 cfu/ml confirmed by plate
count. Microorganisms were incubated statically at room
temperature in the dark and at appropriate time intervals
aliquots were plated onto LB agar and plate counts and colony
morphology were determined. The preliminary results after
approx. 6 months sampling suggest that *V. cholerae* N16961 can
persist under these conditions with only 1-log decrease in
viability. Importantly, N16961 was able to switch from the
smooth (EPSoff) to rugose (EPSon) phenotype at high frequency (up
to 16%) under these conditions by day 50. An advantage of this
study is that it closely mimics a true environmental scenario and
the switching of a wildtype strain of N16961. While these
studies could be extended, these results suggest that *V. cholerae*
can shift to the rugose phenotype in natural environments.

Compositional analysis of the rEPS in *V. cholerae*

[0073] The previous findings above from the laboratory of the
present inventor were the first to report the rugose phenotype in
a classical (6th pandemic) biotype strain of *V. cholerae*. In
order to determine the structural composition of the rugose
variant of classical biotype strain NCTC 6585 and to compare it
to polysaccharides in other strains, a rugose colony of classical

biotype strain NCTC 6585 was inoculated into APW#3 and incubated
at 37°C for 3 days under static conditions to promote EPS
production and biofilm formation. To harvest the EPS, the
cultures were filtered using a large (10 μm) pore size filter
(VWR). The biofilm was washed once gently with PBS to remove
planktonic cells, transferred to a fresh tube and 3 mm glass
beads added to disrupt the biofilm. The sample was then
centrifuged at 20,000 rpm (50,000 x g) for 16 h at 4°C to remove
cell debris and other contaminants. The supernatant was passed
though a Detoxi-Gel Affinitypak column (Polymixin B immobilized
on agarose column) (Pierce) to remove any traces of LPS from the
sample and DNase and RNase (final conc. 100 μg/ml) was added,
then incubated at 37°C for 4 h. Proteinase K (final conc. 100
μg/ml) was added and incubated at 37°C overnight followed by 60°C
for 15 min. Following the addition of 3 volumes of 95% ethanol,
the mixture was precipitated overnight at 4°C then centrifuged at
12,000 rpm for 20 min. The precipitated EPS was washed twice,
first with 80% ethanol and then with 95% ethanol. The EPS
precipitate was resuspended in 0.5 ml MQ, incubated at -80°C for
2 h, lyophilized for 4 h, then analyzed by combined gas
chromatography/mass spectrometry (GC/MS) and performed by the
Complex Carbohydrate Research Center in Atlanta, Georgia.

[0074] The analysis in Table 2 below shows that the rugose EPS
(rEPS) for 6th pandemic strain NCTC 6585 differs markedly from
the EPS of 7th pandemic strain 92A1552 which has glucose as the
predominant sugar (Yildiz et al., 1999) and strain TSI-4 which
has mannose as the predominant sugar (Wai et al., 1998). The
compositional analysis result also suggests that the
extracellular carbohydrate described here is quite different from
O1 LPS which typically contains large amounts of perosamine and
quinovosamine (Raziuddin, 1980). In contrast to the results of
El Tor strain 92A1552 in which 4-linked galactose and 4-linked

glucose were the dominant linkages (Yildiz et al., 1999), the glycosyl linkage analysis using gas chromatography-mass spectrometry (GC-MS) performed on the classical biotype strain show that the predominant linkage is a 4-linked galactosyl residue and may represent the backbone of the saccharide.

TABLE 2. Glycosyl composition and linkage analysis of rEPS from strain NCTC 6585

Sugar	Glycosyl Composition	Glycosyl linkage	
	%	Glycosyl residue[a]	%
Rhamnose	8.92	terminal linked –fucosyl residue	9.8
Fucose	10.46	terminal linked –glucosyl residue	7.9
Mannose	4.68	3 linked –glucosyl residue	8.8
Galactose	18.71	2 linked –glucosyl residue	15.0
Glucose	9.29	4 linked –manosyl residue	14.2
GalNAc	16.86	4 linked –galactosyl residue	24.8
GlcNAc	27.65	2,3,4 linked –fucosyl residue	7.7
		2,3 linked –manosyl residue	11.8

[a] All residues are in the pyranose (p) form.

EXAMPLE 2

[0075] *Vibrio cholerae* can switch to a "rugose" phenotype characterized by an exopolysaccharide (EPS) matrix, wrinkled colony morphology, increased biofilm formation and increased survival under specific conditions. The vps gene cluster responsible for the biosynthesis of the rugose EPS (rEPS) is positively regulated by VpsR. Media (APW#3) promoting EPS production and the rugose phenotype was identified and epidemic strains were found to switch at higher frequency than nonpathogenic strains, suggesting this switch and extracellular polysaccharide is important in cholera epidemiology. In the experiments in this example, transposon mutagenesis on a smooth *V. cholerae* strain was used to identify mutants that were unable to shift to the rugose phenotype under inducing conditions to

better understand the molecular basis of the switch. The present
inventor identified vpsR, galE and vps previously associated with
the rugose phenotype, and also identified genes not previously
associated including rfbD and rfbE having roles in LPS synthesis
and aroB and aroK with roles in aromatic amino acid synthesis.
Additionally, a mutation in amiB encoding N-acetylmuramoyl-L-
alanine amidase caused defects in the switch, motility and cell
morphology. It was also found that a gene encoding a novel
regulatory protein termed RocS (regulation of cell signaling)
containing a GGDEF and EAL domain and that is associated with c-
di-GMP levels is important for the rugose phenotype, EPS, biofilm
formation and motility.

MATERIALS AND METHODS

Mutagenesis and screening for genes with roles in the switch to the rugose phenotype

[0076] The previous identification of culture conditions that
promote the switch to the rugose phenotype at high frequency
(HFRP) by the laboratory of the present inventor (Ali et al.,
2002) was exploited in the development of an assay to identify
genes involved in the molecular switch from the smooth to the
rugose phenotype. In previous studies, the laboratory of the
present inventor reported that incubation of cells in a medium
called APW#3 resulted in a high frequency of V. cholerae smooth
N16961 cells (up to ~80%) switching to the rugose phenotype (Ali
et al., 2002). N16961 is a wildtype seventh pandemic (El Tor
strain) isolated in Bangladesh (Levine et al., 1981). To
identify the genes involved in the molecular switch from the
smooth (EPSoff) to the rugose (EPSon) phenotype, mini-Tn5km2
mutagenesis was used (de Lorenzo et al., 1990 and Herrero et al.,
1990). Tn5 is contained on the R6K-based plasmid pUT/mini-Tn5 Kan
(or pUTKm) that is derived from suicide vector pGP704 (Miller et

al., 1988) and can only be maintained in donor strains (e.g., a
λpir lysogen of *Escherichia coli*) that produce the R6K-specified
λpir protein which is an essential replication protein for R6K
and plasmids derived therefrom. It also carries the origin of
transfer, *oriT*, of plasmid RP4 which enables efficient conjugal
transfer. Delivery of the donor plasmid pUTKm into recipient
cells is mediated by the cognate transposase encoded on the
plasmid at a site external to the transposon. An advantage of
this mutagenesis system is the stability of the Tn5 insertion
since the cognate transposase is not carried with the transposon
during transposition. Thus, each mutant has only a single Tn5
insertion to screen.

[0077] *E. coli* S17 λpir (pUT/mini-Tn5 Km) was mated with
smooth N16961 (EPSoff) cells and 14,500 mini-Tn5 mutants from 30
independent conjugations were obtained and were subsequently
stored in wells of microtiter plates. A high throughput screen
for HFRP mutants was performed in which the transposon mutants
were inoculated into 200 μl APW#3 media in wells of microtiter
plates, incubated for 48 h then replica plated onto LB agar and
incubated for 24-48 h after which the colony morphology was
visually examined. Using this approach, 43 mutants operationally
defined as HFRP-negative that did not produce any rugose colonies
were identified. It was further confirmed that these mutants
were stable and defective in switching to the rugose phenotype
under HFRP-inducing conditions by inoculating a colony into 3 ml
APW#3 in glass test tubes and incubating statically for 48 h at
37°C. Sterile glass beads (4 mm diameter) were then added and
the cultures vortexed to disrupt any aggregates of rugose cells.
Appropriate dilutions of each culture were plated on LB agar and
colonies were counted by standard plate count to determine the
total CFU/ml and the frequency of rugose cells. The 43 mutants
identified and tested by these screening methods did not produce

any detectable rugose colonies under rugose-inducing (HFRP)
conditions and were further studied.

Sequencing of transposon insertion sites and identification of disrupted genes

[0078] To identify the transposon insertion site in these
mutants, a non-laborious arbitrary primed PCR method was used
followed by DNA sequencing similar to that described previously
(Bahrani-Mougeot et al., 2002). Briefly, arbitrary PCR was
performed in two steps: in the first reaction, chromosomal DNA of
the mutant was used as a template for PCR using primers reading
out from both sides of the transposon and two arbitrary primers.
These primary reactions yielded numerous amplicons including some
that were derived from the junction of the transposon insertions.
The products of the first-round PCR were purified by Geneclean
and amplified using a second pair of outward transposon primers
external to the first pair and an arbitrary primer corresponding
to the constant region of the original arbitrary primers. This
secondary PCR reaction serves specifically to amplify products of
the first PCR that include transposon junctions. Amplified
fragment ranged between 100- to 800-bp. The products that gave
the strongest bands were from agarose gels and sequenced using
the same transposon and arbitrary primers used in the second-
round PCR. Sequencing was performed using an automated DNA
sequencer (model 373A, Applied Biosystems) using the Prism ready
reaction dye deoxy termination kit (Applied Biosystems) according
to the manufacturer's instructions.

Cloning of vpsR

[0079] The vpsR gene of N16961 was obtained on a 2.61-kb PCR
fragment using PCR primers KAR486 (5'-CGGGATCCCGCTAAGTCAGAGTTTTT
ATCGC-3'; SEQ ID NO:3) and KAR487 (5'-TCCCCGCGGGTCGGTGGTTTTGATCG
TGT-3'; SEQ ID NO:4). The PCR fragment was digested with *Bam*HI

and *SacII*, respectively, and suitably cloned into the low copy vector pWSK29 (Wang et al., 1999), creating plasmid pDK104.

Motility assay

[0080] Motility was determined in a swarm plate assay by measuring the swarm diameter of each zone after stabbing an equal amount of *V. cholerae* cells (grown in LB broth) into LB media containing 0.3% agar and incubation at 37°C for 4 h.

Microscopic analysis of *amiB* mutant strain

[0081] A single 18 h colony on a LB plate from the wildtype N16961 and *amiB* mutant strain DK630 was resuspended in 1 ml PBS and a 50 µl aliquot smeared onto a glass slide, heat fixed then stained with 0.1% crystal violet for 30 sec. The slide was then rinsed with dH$_2$O, dried and cell morphology observed using a Zeiss Axioskop epifluorescence microscope (Carl Zeiss, Inc. NY). The images were acquired using an AxioCam Mrm camera (Carl Zeiss, Inc. NY).

RESULTS AND DISCUSSION

[0082] The laboratory of the present inventor has successfully sequenced and identified the transposon insertion site in 43 V. cholerae mutants that are unable to switch from the smooth to the rugose phenotype. A summary of a BLAST search against the published V. cholerae N16961 genome to identify the disrupted genes (Heidelberg et al., 2000) is shown in Table 3.

TABLE 3. Representative HFRP mutants of *V. cholerae* N16961

Mutant[a]	Locus[b]	Predicted protein	Predicted function
DK568 (2)	VC0243	RfbD	LPS biosynthesis, GDP-mannose 4,6 dehydratase
DK623 (1)	VC0244	RfbE	LPS biosynthesis, perosamine synthase
DK578 (2)	VCA0744	GalE	LPS biosynthesis, UDP-glucose 4-epimerase
DK589 (1)	VC0920	Vps (EpsF)	EPS biosynthesis, glycosyl transferase
DK576 (2)	VC0921	Vps (Wzx)	EPS, polysaccharide export, flippase
DK588 (7)	VC0922	Vps	EPS, hypothetical protein
DK562 (13)	VC0665	VpsR	EPS biosynthesis, σ^{54} transcriptional activator
DK614 (10)	VC2628	AroB	aromatic a.a. synthesis, 3-dehydroquinate synthase
DK625 (1)	VC2629	AroK	aromatic a.a. synthesis, shikimate kinase
DK630 (1)	VC0344	AmiB	N-acetylmuramoyl-L-alanine amidase
DK567 (3)	VC0653	RocS	regulatory, contains GGDEF and EAL domains

[a] Numbers in brackets indicate number of mini-Tn5 mutants having insertions in same locus.
[b] Loci and predicted proteins derived from the *V. cholerae* N16961 TIGR sequencing project.

[0083] Previous transposon mutagenesis studies have identified gene mutations that result in stable rugose-to-smooth mutants (Watnick et al., 1999; Yildiz et al., 1999; and Ali et al., 2000). In contrast, taking advantage of the conditions developed by the laboratory of the present inventor that promote switching to rugose phenotype, transposon mutagenesis on a smooth strain of N16961 was performed and stable mutants that were unable to switch to the rugose phenotype under rugose-inducing conditions were screened. While the findings revealed mutants with defects in genes previously identified with roles in the rugose phenotype such as several biosynthesis (*vps* operon) and regulatory genes (*vpsR*) and LPS genes *(galE)*, this screen also identified mutants sustaining insertions in previously unidentified genes. These

newly identified mutants could be clustered into several functional groups coding for LPS (*rfbD* and *rfbE*) in which the genes might have roles in catalyzing the addition of certain sugar linkages and whereby impairment in the LPS structure might also be linked to the shutdown of the rugose (EPSon) phenotype; genes involved in aromatic amino acid synthesis (*aroB* and *aroK*) whereby aromatic amino acid synthesis genes might be directly or indirectly associated with the rugose phenotype; a gene involved in cell wall hydrolysis (*amiB*) and a novel locus, VC0653, designated "*pdeA*-like" in the N16961 genomic database, which the present inventor has now termed RocS (for regulation of cell signaling; SEQ ID NO:1) encoding a putative protein (SEQ ID NO:2) containing GGDEF and EAL domains. While the exact function of GGDEF and EAL domains is not well understood but are thought to have some role in signaling, proteins containing these domains are widespread in prokaryote species and appear to play a key function in the regulation and biology of many species.

VpsR has an essential role in switching to the rugose phenotype

[0084] The importance in regulating *vps* biosynthetic genes in *V. cholerae* led the present inventor to further study several *vpsR* transposon mutants, designated DK562 and DK581. VpsR, encoded by the locus VC0665 is a predicted 444 amino acid protein with high similarity to the family of σ54 response regulators such as NtrC, AlgB, and HydG (Yildiz et al., 2001 and Ali et al., 2000). The laboratory of the present inventor found that supplying *vpsR* on plasmid pDK104, can restore switching to the rugose phenotype in both these *vpsR* mutants. These findings confirm that the defect in switching to the rugose phenotype in these mutants is due to the mutation in *vpsR*. Since VpsR is predicted to be a transcriptional activator, the present inventor speculated whether it controlled motility in *V. cholerae*. The

motility tests performed on the VpsR mutants (DK562 and DK581) showed that the mutants are consistently ~50% reduced in its motility compared to the parent N16961 (data not shown). Since *V. cholerae* cells are typically motile and motility is important for virulence (Yancey et al., 1978 and Richardson, 1991), it is tempting to speculate that VpsR might also have a role in virulence of *V. cholerae*. Although VpsR is important in regulating EPS (*vps*) biosynthesis genes and potentially other phenotypes, the conditions promoting VpsR expression and its mechanism of regulating *vps* genes is not well understood.

The AmiB amidase has a role in the switch to the rugose phenotype

[0085] The AmiB (N-acetylmuramoyl-L-alanine amidase) protein is encoded by the *amiB* gene (VC0344). Since it was previously found that rugose strains of *V. cholerae* are affected in motility (Ali et al., 2002), *amiB* mutants were tested to see if their motility was affected. Motility assays showed that the AmiB mutant (strain DK630) was consistently >50% reduced in its motility (10 mm zone) compared to its parent N16961 (26 mm zone) (Fig. 3). These results suggest that AmiB affects motility and the rugose phenotype in *V. cholerae*.

[0086] The bacterial cell wall is typically composed of a heteropolymer known as murein or peptidoglycan. Many Gram-negative bacteria degrade up to 50% of their murein per generation and recycle it to form new murein (Goodell, 1985; Park, 1993). N-acetylmuramoyl-L-alanine amidases are often associated with autolysis or microbial cell wall hydrolysis. Surprisingly, enzymes in Gram-negative bacteria that cleave the septum such as AmiB have only recently been studied in a few species, and in *E. coli*, AmiB mutants are found growing as long chains of unseparated cells (Heidrich et al., 2001 and Holtje et al., 2001). In *Azotobacter vinelandii*, an N-acetylmuramoyl-L-

alanine amidase is linked to alginate production by the ability
of *A. vinelandii* cells to recycle their cell wall (Nunez et al.,
2000).

[0087] A BLAST search shows that the *V. cholerae* AmiB sequence
has high similarity to N-acetylmuramoyl-L-alanine amidases found
in a wide variety of species including *Pseudomonas aeruginosa*
($7e^{-78}$), *Salmonella enterica* Typhi ($7e^{-69}$), *E. coli* O157:H7 ($6e^{-57}$)
and *Yersinia pestis* ($6e^{-50}$). AmiB in *V. cholerae* strain N16961 is
predicted to be a 59-kDa protein that is unusually rich in serine
(9.5%), proline (6%) and threonine (6%). Such a composition is
common in protein domains associated with the cell wall in Gram-
positive bacteria (Fischetti et al., 1991) and is similar to a
putative peptidoglycan hydrolase of *Lactococcus lactis* (*acmB*)
(Huard et al., 2003). In *V. cholerae*, like in *E. coli* and *Y.*
pestis, *amiB* is located immediately upstream of *mutL* which has a
role in DNA mismatch-repair (Tsui et al., 2003 and Parkhill et
al., 2001). A computer analysis using PSORT shows that *V.*
cholerae AmiB is predicted to have a cleavable N-terminal signal
sequence and an analysis using TMpred strongly predicts that AmiB
has two transmembrane domains (score 2363), one at the N-terminal
end (a.a. 10-29) which could also represent an N-terminal signal
anchor sequence and another transmembrane domain at the C-
terminal end (a.a. 446-465). One would expect TMpred to predict
a transmembrane region at the N-terminal end if a sec-dependent
signal sequence was also predicted. The *V. cholerae* AmiB is also
predicted to contain a LysM (lysin motif) domain at its C-
terminal end and this has been found in enzymes involved in cell
wall degradation (Bateman et al., 2000). Interestingly, the *V.*
cholerae AmiB contains a Arg-Gly-Asp (RGD) motif that is often
associated with a surface binding domain for various mammalian
adhesion proteins.

[0088] Since AmiB has been associated with septation in other species such as *E. coli* (Heidrich et al., 2001 and 2002), it was determined whether the *V. cholerae* AmiB mutant was affected in its cellular morphology as well as the rugose phenotype. Examination of the cells showed an obvious difference between the AmiB mutant (Fig. 4B) and the wildtype strain (Fig. 4A) in the morphology and arrangement of the cells. Many cells of the AmiB mutant were altered in shape and some were dramatically increased in cell size (length and width). The AmiB mutant appeared to have a higher percentage of cells in chains. This finding suggests that cell division or septation might be affected. No difference in growth rate between wildtype and the AmiB mutant DK630 was found (data not shown), suggesting that the difference in cell structure is not due to differences in growth rate. While the findings of cells grown on LB plates bred true following subculture, no obvious dramatic differences between the strains when grown in LB broth were found (data not shown). Although further studies are required to analyze the cellular structure and morphology of the AmiB mutant in more detail, such as using electron microscopy, the results of the studies presented in this example suggest there is a link between cell division, structure or septation and the rugose phenotype of *V. cholerae*. These findings provide evidence for a new function for a prokaryotic amidase, namely its importance in the switch to the rugose phenotype and biofilm formation.

V. cholerae RocS: a conserved regulatory protein with a GGDEF and EAL domain regulates the rugose phenotype

[0089] Another class of mutants that the laboratory of the present inventor was particularly interested in were mutants with defects in the locus VC0653 encoding a putative protein termed RocS (formerly "PdeA-like" protein in the database) containing a GGDEF and EAL domain. It is important to note that three

independent mutants containing mutations in rocS from three
independent conjugations were isolated. This result suggests
that V. cholerae RocS has an important role in rEPS production,
the rugose phenotype, in biofilm formation and possibly other
phenotypes. The defect in the rugose phenotype in this mutant
was not explained by differences in growth rate between the
wildtype N16961 and RocS (DK567) cells (data not shown). The
finding that V. cholerae RocS mutants appear to be defective in
the switch to the rugose phenotype prompted testing their
motility as described above. Motility assays showed that the
RocS mutant (DK567) was consistently >50% reduced in its motility
(12 mm zone) compared to its parent N16961 (26 mm zone)
suggesting that this locus also affects motility in V. cholerae
(Fig. 3). Based on these results, the present inventor proposes
that V. cholerae RocS (and c-di-GMP) regulates several
phenotypes, including those with roles in virulence, biofilms and
the persistence of species.

[0090] Interestingly, GGDEF domains have been shown in
proteins known to be involved in the regulation of cellulose (β-
1,4-glucan) synthesis (Ausmees et al., 2001). Cellulose
production in Acetobacter xylinum, Rhizobium leguminosarum bv.
trifolii and Agrobacterium tumefaciens is modulated by the
opposing effects of two enzymes, diguanylate cyclase (Dgc) and c-
di-GMP diesterase (PdeA), each controlling the level of the novel
signaling molecule c-di-GMP in the cell (Amikam et al., 1989 and
Ross et al., 1990 and 1991). Diguanylate cyclase acts as a
positive regulator by catalyzing the formation of c-di-GMP that
specifically activates cellulose production while the
phosphodiesterase cleaves c-di-GMP and negatively regulates
cellulose. The c-di-GMP molecule is predicted to be a
reversible, allosteric activator (effector) of cellulose
biosynthesis (Ross et al., 1991). Furthermore, genetic

complementation studies using genes from different species
encoding proteins with GGDEF domains as the only element in
common suggest that the GGDEF domain has a role in diguanlylate
cyclase activity and is important in modulating the level of c-
di-GMP (Ausmees et al., 2001). The laboratory of the present
inventor found that the *V. cholerae* RocS protein plays a key role
in the switch to the rugose phenotype that is associated with the
production of an EPS-like material and increased biofilm
formation.

[0091] A BLAST search of the *V. cholerae* RocS shows that it is
highly conserved and has significant homologues to putative
proteins found in a wide variety of other species including *P.
aeruginosa* (PA0575; 42% id; $5e^{-92}$), *Bacillus anthracis* (BA5593;
37% id; $6e^{-90}$), *Ralstonia solanacearum* (RSc0588; 36% id; $4e^{-88}$) and
A. xylinum (c-di-GMP diguanylate cyclase Dgc; 40%, $9e^{-82}$).
Although *dgc* and *pdeA* genes share some homology and have similar
domain architecture, the finding here that the *rocS* mutant is
unable to produce an EPS-like material is more consistent with a
diguanlylate cyclase *(dgc)* mutant that is unable to produce
cellulose. *V. cholerae* RocS has slightly higher similarity to *A.
xylinum* Dgc than PdeA (data not shown). Recent reports have
identified "RocS" homologues in *P. aeruginosa* that appear
essential for biofilm formation (Connolly et al., 2003) and in
V. parahaemolyticus that regulate capsular polysaccharide
production (Gùvener et al., 2003). Additionally, the
autoaggregation phenotype (which is typical of the rugose
phenotype) in the plague bacterium *Y. pestis* requires the GGDEF-
containing protein HmsT (Jones et al., 1999). Although
homologous regulatory (GGDEF-containing) proteins have been found
in several species and have been associated with wrinkled
colonies, EPS production and biofilm formation, their role in
regulating these processes has not been well studied, in part due

to the lack of available reagents. There is growing evidence
suggesting that GGDEF-containing proteins possess nucleotide
cyclase activity (Ausmees et al., 2001; Ross et al., 1987; Pei et
al., 2001 and Tal et al., 1998) and are widespread in bacteria
(Croft et al., 2000 and Galperin et al., 2001). The potential
widespread occurrence of this protein homologue and c-di-GMP in
prokaryotes suggests common regulatory systems and that they
might have an important function in regulating phenotypes
including EPS production, the rugose phenotype and biofilm
formation in *V. cholerae* and phenotypes in other species.

[0092] A rugose-like phenotype has been reported in several
species including *S. enterica* Enteritidis (Petter, 1993), *S.
enterica* Typhimurium (Anriany et al., 2001), *V. parahaemolyticus*
(Güvener et al., 2003), *P. aeruginosa* (D'Argenio et al., 2002)
and *Enterobacter sakazakii* (Farmer et al., 1980). It is now
becoming increasingly recognized that the rugose phenotype might
have an important role in *V. cholerae* and in several other
species suggesting that these "variants" might represent the "tip
of an iceberg". Data is accumulating suggesting that rugose
variants are filling a specific role in biofilm formation,
particular niches or in particular environments. In the case of
V. cholerae, rEPS production, the rugose phenotype and HFRP may
provide some evolutionary or adaptive advantage to that
subpopulation of cells in a particular environment.

[0093] In the studies presented in this example, the
identification by the laboratory of the present inventor of
conditions that promote the high frequency switch from the smooth
to the rugose phenotype of *V. cholerae* was exploited to identify
and study the genes involved in the molecular switch between the
smooth and rugose phenotypes. It appears that some *V. cholerae*
strains have evolved an efficient mechanism for a high frequency
shift to the rugose phenotype that is associated with EPS

production, increased biofilm formation and increased cell
survival under specific conditions. The switching ability that
can lead to increased biofilm formation might provide an adaptive
advantage in particular environments where strains compete for
the same ecological niche and where slight variations in
phenotype that promote resistance, increased colonization or cell
aggregates are important for adaptation and survival.

EXAMPLE 3

[0094] *Staphylococcus aureus* is an important pathogen of
humans and animals and its antibiotic resistance is a public
health concern. Biofilm formation is essential in pathogenesis,
and the ability to form biofilms and resist traditional
antibiotic treatments often results in difficult-to-treat and
persistent infections. As such, novel antimicrobial approaches
are of great interest to the scientific, medical and agricultural
community. The present inventor proposed in Example 1 that
modulating levels of the cyclic dinucleotide signalling molecule,
c-di-GMP (3', 5'-cyclic diguanylic acid), has application in
regulating phenotypes of prokaryotes. In the experiments
described below in this example, extracellular c-di-GMP shows
activity against human clinical and bovine intramammary mastitis
isolates of *S. aureus*, including methicillin-resistant (MRSA)
strains. The present example shows that chemically synthesized
c-di-GMP is soluble and stable in water physiological saline and
stable following boiling and exposure to acid and alkali.
Treatment of *S. aureus* with extracellular c-di-GMP inhibited
cell-to-cell (intercellular) adhesive interactions in liquid
media and reduced (>50%) cell-to-surface interactions and biofilm
formation in human and bovine isolates compared to untreated
controls. c-di-GMP inhibited the adherence of *S. aureus* to human
epithelial HeLa cells with c-di-GMP showing no obvious toxicity

on HeLa cells and was non-lethal in mice. The guanosine
nucleotide analog cGMP had a lesser effect on biofilms while 5'-
GMP had no effect. The data here suggests that *S. aureus* might
sense and respond to extracellular c-di-GMP signals. Cyclic
dinucleotides, such as c-di-GMP, when used either alone or in
combination with other antimicrobial agents, represent a novel
and attractive approach in inhibiting and controlloing biofilms
and in the control and treatment of infection.

MATERIALS AND METHODS

[0095] **c-di-GMP, cGMP and 5'-GMP nucleotides used.** c-di-GMP
used in the studies in this example was biochemically synthesized
in pure form using a recently described novel synthesis method
that produces a pure and high yielding preparation of c-di-GMP
diammonium salt (Fig. 5A) (Hayakawa et al., 2003). Before use,
the purity and stability of lyophilized c-di-GMP was determined
by first resuspending the molecule in 0.9% NaCl creating a 2 mM
solution then confirmed by HPLC analysis and ESI-TOF MS
spectrometry. The cGMP (guanosine 3', 5'-cyclic monophosphate,
Sigma #G7504) and 5'-GMP (guanosine 5'-monophosphate, TCI, Tokyo
Kasei Kogyo Co.) nucleotides were also used. Unless otherwise
stated, a 4 mM stock solution of each nucleotide in 0.9% NaCl was
prepared and stored at 4°C until needed.

[0096] **Bacterial strains and growth media used.** Human
clinical isolates of *S. aureus* were used in this study and stored
at -70°C in 50% glycerol. *S. aureus* strain DK825 was isolated in
2003 from the blood culture of a patient at the Veterans Affairs
Medical Center (approximately 200 acute care beds) in Baltimore,
MD. DK825 was isolated by the diagnostic microbiology laboratory
at the VAMC and confirmed as methicillin-resistant *S. aureus*
(MRSA) using standard plating techniques, latex agglutination
using a BactiStaph Latex 150 test kit (Remel) for the detection

of coagulase and by antibiotic susceptibility tests (Sensititre
plates, Microbiology Systems). *S. aureus* strain 15981 is a
highly adherent hyperbiofilm human clinical strain that was
isolated in 1999 from an otitis patient by the clinical
laboratory at the Universitaria de Navarra, Spain (Iñigo Lasa,
personal communication) (Valle et al., 2003). *S. aureus* 15981 is
a natural *agr* mutant and is susceptible to MET, AMX, CLI, ERY,
DOX, FOF, VAN, CIP and resistant to GEN. The wildtype bovine
subclinical mastitis strains used in this study were V329
(hyperbiofilm strain) (Cucarella et al., 2001), V299 (*bap*-
negative *icaADBC*-positive) (Cucarella et al., 2004) and V315
(*bap*-negative and *icaADBC*-negative) (Cucarella et al., 2004). *S.
aureus* strain M556 (isogenic transposon insertional *bap*-mutant of
V329) was also used (Cucarella et al., 2001). Unless otherwise
noted, *S. aureus* strains were grown at 37°C on sheep blood agar
plates or in tryptic soy broth (TSB, Difco) supplemented with
0.25% glucose.

[0097] **c-di-GMP stability tests.** Although the term c-di-GMP
is used here, c-di-GMP diammonium salt (and not the free
diphosphoric acid) was used for these stability tests. (i) In
boiling water: A 2 mM solution of c-di-GMP in water was prepared
by dissolving 2.42 mg (3.3 µmol) of c-di-GMP in 1.65 mL of ion-
exchanged/ion-free water (prepared by passing distilled water
through ion-exchange resins). This solution was heated at 100°C
for 10 min and then concentrated under reduced pressure. The
resulting residual was subjected to the HPLC analysis under
conditions shown below. (ii) In pH 3 solution: From the 2 mM c-
di-GMP aqueous solution described above, 500 µl (containing 1 µM
of c-di-GMP) was dissolved in 20 mL of 1 mM HCl to produce a
solution with pH 3 (confirmed by pH meter). The resulting
solution was stirred at room temperature for 1 h and then
neutralized by the addition of 200 mL of a 0.1 mM NaOH. Water

was evaporated under reduced pressure and the resulting residue was subjected to HPLC analysis. (iii) In pH 10 solution: From the 2 mM c-di-GMP aqueous solution described above, 500 μl (containing 1 μM of c-di-GMP) was dissolved in 200 mL of a 0.1 mM NaOH aqueous solution to produce a solution with pH 10 (confirmed by pH meter). The resulting solution was stirred at room temperature for 1 h. The reaction was quenched by addition of 20 mL of a 1 mM HCl. Concentration of the resulting neutral solution under reduced pressure gave a residual material that was subjected to the HPLC analysis. The HPLC analysis was performed on a Waters 2695 Separation Module with a Waters 2996 Photodiode Array Detector under the following conditions. Column: Nacalai Tesque COSMOSIL 5C18-AR-II column (4.6 mm (diameter) x 250 mm (length)); detection: 254 nm of UV ray; temperature: 40°C; eluent: A = 0.9% NaCl (aq), B = a 20:80 mixture of water:acetonitrile; flow rate:1 mL/min; 0-10 min: A 100%; 10-60 min: linear gradient from A100%/B 0% to A 40% to B 60%.

[0098] **Antibiotic susceptibility testing.** Susceptibility tests were performed in Sensititre microtitre plates according to the manufacturer's instructions (Microbiology Systems).

[0099] **Effect on growth rate of _S. aureus_.** _S. aureus_ DK825 was subcultured from glycerol stocks onto a blood agar plate and incubated at 37°C for 18 h. A single colony was then inoculated into 5 ml TSB broth (supplemented with 0.25% glucose) and incubated at 37°C for 24 h with shaking at 250 rpm. From a 10^{-3} dilution of the overnight culture a 100 μl aliquot was inoculated into tubes containing 5 ml TSB broth resulting in an initial cell count of 10^5 cfu/ml (confirmed by plating). For "treated" samples, an appropriate aliquot of c-di-GMP was added to give 200 μM c-di-GMP (final concentration). As a negative "untreated" control, tubes containing TSB and a similar volume of 0.9% NaCl were included in the studies. At time zero, 50 μl from each

sample was plated on blood agar plates to determine the initial
cfu/ml. The tubes were then incubated at 37°C for 8 h under
shaking conditions with aliquots being plated from all tubes
every 30 min.

[00100] **Tube agglutination assay and light microscopy.**
Colonies from a 18 h blood agar plate were inoculated into 1 ml
PBS to make a 0.5 McFarland standard containing ~5×10^8 cfu/ml
(confirmed by plate count). A 5 µl aliquot from the 0.5
McFarland standard was then inoculated into 5 ml polystyrene
tubes containing 1 ml TSB representing ~10^5 cfu/ml (confirmed by
plate count). These tubes were inoculated either with 50 µl c-
di-GMP to give a 200 µM c-di-GMP final concentration representing
a "treated" sample or inoculated with 50 µl 0.9% NaCl as a
control and representing an "untreated" control. Cultures were
incubated at 37°C for 24 h statically. Following incubation,
these cultures were examined macroscopically and microscopically
(Zeiss Axioskop) for the presence or absence of visible cell-to-
cell clumping.

[00101] **Effect of c-di-GMP on *S. aureus* biofilm formation.** *S.
aureus* strains were subcultured from glycerol stocks onto blood
agar plates and incubated at 37°C for 18 h. A single colony was
inoculated into 5 ml TSB broth with a sterilized loop and
incubated at 37°C for 18 h with shaking at 250 rpm and until the
O.D.$_{660}$ reached 3.0 as measured spectrophotometrically using a
spectrophotometer (SpectraMAX 250, Molecular Devices). Following
incubation, the culture was diluted 1:250 with fresh TSB and a
200 µl of the diluted culture was transferred into wells of a
flat-bottom polystyrene microtitre plate (Evergreen Scientific).
To test the effect of c-di-GMP treatment on biofilm formation, a
series of "treated" samples containing 10-fold dilutions of c-di-
GMP were set up in TSB which contained the following final
concentrations of c-di-GMP (0-, 2-, 20- and 200 µM). In these

biofilm experiments, similar volumes of 0.9% NaCl was added to a set of different wells representing the "untreated" control samples. The microtitre plates were then incubated statically at 37°C for 24 h or 48 h. Following incubation, the supernatant was carefully discarded and the wells washed twice with 260 μl PBS. The plate was then kept for drying on a paper towel for 30 min after which 260 μl 0.1% crystal violet was added to each well and the plates incubated at room temperature for 30 min. The crystal violet was discarded and plate washed gently with water, the wells allowed to dry for 30 min then 260 μl DMSO was added to each well and gently agitated for 1 h and the O.D.$_{570}$ measured by using a spectrophotometer (SpectraMAX 250, Molecular Devices). The results of these biofilm assays were based on data obtained from at least three independent colonies tested in duplicate.

[00102] **Effect of c-di-GMP on *S. aureus* pre-formed biofilms.** *S. aureus* DK825 was subcultured from glycerol stocks onto blood agar plate and incubated at 37°C statically. A single colony was inoculated into 5 ml TSB broth (containing 0.25% glucose) and incubated at 37°C overnight with shaking at 250 rpm until the culture reached OD$_{660}$ ~ 3.0. Following incubation the culture was diluted to 1:250 with fresh TSB broth and a 200μl of the diluted culture was transferred into each well of microtitre plate and the plate containing wells with *S. aureus* cultures was incubated statically at 37°C for 24 h. After 24 h, an appropriate volume of c-di-GMP was added to give a final concentration of 200 μM and represented the "treated" sample. As an "untreated" control, an identical volume of 0.9% NaCl was added to independent wells. The plates were then incubated statically at 37°C for an additional 24 h. Following incubation the culture was discarded and microtitre plate was washed twice with 1X PBS. An equal volume (260 μl) of 1X PBS was added to each well for washing. The plates were then kept for drying on paper towel ~30 min. A

260 μl of 0.1% crystal violet was added to each well to stain the cells in the biofilm and the plates were incubated at room temperature for 30 min. Crystal violet was discarded and plate was washed with tap water gently and kept on a paper towel to dry for 30 min. A 260 μl DMSO was added to each well and gently rocked for 1 h. To quantitatively assay the amount of biofilm, the OD_{570} was measured by a spectrophotometer (SpectraMAX 250, Molecular Devices). The results were based on at least three independent colonies tested in duplicate.

[00103] **Epithelial cell assay.** HeLa cells (ATCC CCL2) were grown to confluence in complete medium (10% FBS from Sigma, DMEM/F12 with glutamine from Invitrogen and 50 μg/ml gentamicin) and were trypsinized with 0.1% trypsin-EDTA. Approximately 1×10^5 HeLa cells were seeded in each well of a chamber slide and then the HeLa cells were incubated at 37°C in 5% CO_2 for at least 18 h until 85% confluent. Prior to infection, the HeLa cells were washed twice with warm PBS and then 500 μl warm FMEM/F12 was added to each well. For the bacterial adherence assay, an overnight culture of *S. aureus* strain DK825 was grown overnight in 5 ml LB broth at 37°C with shaking at 250 rpm. Following incubation, 1 ml of the overnight culture was pelleted, washed twice with PBS and resuspended in 1ml of PBS. The HeLa cells in the wells were washed twice with 500 μl of HBSS and aliquots of DMEM with the desired final concentration of c-di-GMP (0-, 2-, 20- and 200 μM) were prepared. DMEM (500 μl) containing the respective concentrations of c-di-GMP was inoculated into the wells containing HeLa cells followed by the addition of 10 μl ($\sim 10^7$ cells) *S. aureus* (MOI HeLa:bacteria$\sim$1:100). The epithelial cell assay was incubated in CO_2 for 45 min. Following incubation the cells were washed twice with 500 μl PBS and fixed with 2% formalin for 20 min. The cells were again washed twice with PBS and stained with Giemsa stain for 10 seconds, washed three times

with PBS, then the cells covered with 40 μl of 90% glycerol in
water and a cover slip was placed. The slides were observed
under a light microscope (Zeiss Axioskop) at 630 X mag. The
level of bacterial adherence to 100 individual HeLa cells was
calculated for each duplicate treatment and the average
determined.

[00104] **Safety and toxicity assays**. For HeLa cell toxicity
studies, various concentrations (0-, 25-, 50-, 100-, 200- and 400
μM) of c-di-GMP were tested in separate wells of chamber slides
containing HeLa cells prepared as described above in complete
media (in the absence of bacteria). HeLa cell morphology was
examined microscopically after 12-, 24- and 48 h incubation. The
potential lethality of c-di-GMP administered to mice was also
examined. In these studies, adult female CD-1 mice and 5 day old
infant mice were inoculated orally with 50 μL of 200 μM c-di-GMP
and examined 24 h after c-di-GMP treatment.

RESULTS AND DISCUSSION

[00105] The ability of *S. aureus* to form biofilms on various
surfaces such as medical devices and tissues is an important and
necessary first step in the pathogenesis of disease. The overall
prevalence of *S. aureus* in the community and in hospitals, its
ability to form biofilms, and the fact that *S. aureus* is
frequently resistant to multiple antibiotics makes *S. aureus* a
major public health problem. As such, novel intervention and
antimicrobial methods that reduce biofilm formation in humans and
animals may result in a corresponding increase in antibiotic
susceptibility and more effective prevention and treatment
strategies. This study shows that extracellular c-di-GMP
inhibits cell-to-cell interactions and biofilm formation in human
and animals isolates of *S. aureus*.

[00106] **Identification of GGDEF domains in *S. aureus* suggests a
link to c-di-GMP.** c-di-GMP is associated with proteins
containing GGDEF amino acid domains. The GGDEF domains are ~180
amino acid protein fragments that have an adenylate cyclase-like
fold and work as a cyclic diguanylate synthetase. These domains
have a conserved GG(D/E)EF motif (SEQ ID NO:5) but also many
other conserved residues (Galperin, 2001 and 2004). GGDEF
proteins are increasingly being found to be important in the
regulation of bacterial exopolysaccharide, biofilm formation,
colonization and adherence (Bomchil et al., 2003; D'Argenio et
al., 2002; Jones et al., 1999 and Römling et al., 2000). These
types of protein are widespread in bacteria suggesting that a
broad range of species can potentially be targeted and phenotypes
regulated by modulating c-di-GMP levels and numerous species have
a large number of proteins with GGDEF domains (Galperin, 2001
and 2004). Interestingly, a search of the COG database shows
that *S. aureus* has only one protein (SA0701, COG2199) with a C-
terminal GGDEF domain and another protein (SA0013, COG3887) with
a modified GGDEF domain (Tatusov et al., 2001). According to a
Pfam analysis (pfam.wustl.edu), the N-terminal fragment of SA0701
is predicted to be an integral membrane sensor domain of the 5TM-
5TMR_LYT type (5 predicted transmembrane segments, Pfam entry
PF07694) and therefore is predicted to be a membrane receptor
with a diguanylate cyclase output domain. Unfortunately, the
role of these putative signal transduction proteins in *S. aureus*
and whether they are potentially linked to c-di-GMP, whether c-
di-GMP is made by *S. aureus* and whether the regulatory effects of
c-di-GMP are similar in all species is not yet known.

[00107] **Stability of c-di-GMP.** The stability of c-di-GMP under
various physical conditions and treatments is not well
understood. However, if c-di-GMP is to be used as part of an
antimicrobial strategy or therapeutic agent, its stability needs

to be better studied. The laboratory of the present inventor
determined the stability of c-di-GMP after several storage
conditions and following various exposures including heat, acid
(pH 3) and alkali (pH 10) treatment.

[00108] HPLC analysis of a neat (from lyophilized powder) form
of c-di-GMP that was resuspended in ion-free distilled water
(prepared by passing distilled water through an ion-exchange
resin column) immediately before HPLC analysis to produce a 2 mM
stock indicated that storage of a neat form of c-di-GMP for
several days at -78°C formed aggregate molecules whose structure
is unknown at present but is being determined (data not shown).
Furthermore, storage of a 2 mM stock solution having water as the
diluent at ambient temperature (10-20°C) for several days
resulted in the formation of an aggregated product.
Interestingly, however, adjustment of the solution to a
concentration of 0.9% NaCl was found to revert the aggregated
molecules back to the monomeric form as determined by HPLC and
ESI-TOF MS spectrometry (data not shown). In phosphate buffer
solution, c-di-GMP was stable in a 100 mM phosphate buffer for at
least one month at -78°C, 4°C, and 25°C and did not undergo any
structural changes (data not shown). In a 0.9% NaCl solution,
HPLC analysis showed that c-di-GMP was very stable as the
monomeric structure following storage at either -78°C, 4°C, and
25°C for at least three months (Fig. 5B). c-di-GMP was also
found to be stable in a 100 mM ammonium acetate buffer for at
least one month at -78°C, 4°C, and 25°C and did not undergo any
structural changes. These results suggest that stock solutions
of c-di-GMP should be prepared in 0.9% NaCl such that c-di-GMP
will be stable and remain as a monomeric form for at least
several months.

[00109] Consistent with a previous study by Ross et al. (Ross
et al., 1991) in G. xylinum examining the role of c-di-GMP in

activating cellulose production, HPLC analysis in this study demonstrated that chemically synthesized c-di-GMP is stable following 10 min exposure at 100°C. While Ross found that the "activator" (measured by cellulose activating activity) is labile after treatment in relatively strong alkali (0.2 N NaOH, ~pH 13.5, 37°C for 24 h), HPLC analysis conducted in this study suggests that c-di-GMP is stable following treatment in mild alkali (0.0001 N NaOH, pH 10, 20-25°C for 1 h). Consistent with the findings in the previous study by Ross et al. (Ross et al., 1991), the data from the laboratory of the present inventor showed that chemically synthesized c-di-GMP is stable following acid treatment (0.001 N HCl, pH 3, 20-25°C for 1 h). Based on these studies, c-di-GMP is a stable and soluble low molecular weight molecule.

[00110] **Antibiotic susceptibility of S. aureus strain DK825.**
To further characterize S. aureus DK825, antibiotic susceptibility tests were performed against commonly used antibiotics. Antibiotic susceptibility tests for DK825 produced the following MIC profiles (μg/ml): Penicillin, PEN >8; Ampicillin, AMP 4; Oxacillin, OXA >2; Tetracycline, TET >32; Rifampin, RIF >2; Clarithromycin,CLR >4; Levofloxacin, LVX 2; Ciprofloxacin, CIP >2; Moxifloxacin, MXF 2; Clindamycin, CLI >2; Erythromycin, ERY >4; Vancomycin, VAN <0.5. These findings further showed that S. aureus DK825 is resistant to multiple commonly used antibiotics but is sensitive to vancomycin.

[00111] **c-di-GMP treatment prevents S. aureus cell-to-cell interactions.** Initial experiments on the effect of c-di-GMP on S. aureus examined whether c-di-GMP had any effect on the growth rate of S. aureus. Hourly examination of the growth rate for up to 8 h showed that 200 μM c-di-GMP had no obvious effect on growth rate. The laboratory of the present inventor then tested whether c-di-GMP treatment affected the macroscopic growth and

appearance of *S. aureus* cells after 24 h static incubation in liquid culture. Following incubation, the treated and untreated cultures were visually examined for visible cell-cell clumping and aggregation. The results with *S. aureus* DK825 showed that the 200 μM c-di-GMP treated culture exhibited no obvious visible cell aggregation or pellet at the bottom of the tube (Fig. 6A) while the untreated culture showed obvious cell aggregation and a pellet (Fig. 6B). Plating of c-di-GMP-treated and untreated cultures showed no difference in final cell count (6×10^8 CFU/ml) between the cultures further suggesting that the inhibition of cell-to-cell interactions is not due to major differences in growth rate or final cell numbers. Similar effects on cell aggregation at the bottom of the tube in response to c-di-GMP were observed with several independent wildtype *S. aureus* bovine mastitis strains (V329, V299, V315) (data not shown). A recent study by Cucarella *et al.* (Cucarella et al., 2001) reported that accumulation of cell aggregates at the bottom of the tube was macroscopically only observed for wildtype V329 and not with the isogenic *bap* mutant M556. While much less visible clumping in strain M556 was observed, the results here clearly suggested that c-di-GMP treatment inhibits cell aggregation of M556 cell at the bottom of the tube compared to untreated cultures (Figs. 6E and 6F). It is also important to note that strain M556, while being a *bap* mutant is *ica*-positive (Cucarella et al., 2001). Two possibilities that might explain the findings in these two studies are that the results of Cucarella *et al.* were based on shaking cultures or that TSB obtained from different sources might influence cell growth and cell interactions. Inhibition of cell aggregation was consistent and was observed following similar c-di-GMP treatment in which the c-di-GMP used was independently synthesized. The results from this macroscopic analysis indicate that *S. aureus* respond to extracellular c-di-

GMP and that c-di-GMP treatment inhibits *S. aureus* cell aggregation in human and animal isolates.

[00112] In order to further study the basis underlying the macroscopic difference observed between treated and untreated cultures described above, the cells in these cultures were vortexed, Gram-stained and visualized by light microscopy. The name *Staphylococcus* is derived from the Greek meaning "bunch of grapes". However, Gram-stained examination of the DK825 c-di-GMP treated cultures showed dramatically less cell-to-cell interactions and clumping in liquid media (Fig. 6C) than untreated cells that showed typical grape-like clusters (Fig. 6D). Similar to the findings in Fig. 6 and consistent with the macroscopic analyses, less intercellular interactions and clumping was observed by Gram-stain and light microscopy for the wildtype bovine mastitis strains (V329, V299, V315) (data not shown). It is important to note that wildtype strain V329 is *bap*-positive *icaADBC* positive while wildtype strains V299 and V315 are *bap*-negative *icaADBC* positive and *bap*-negative *icaADBC* negative, respectively (Cucarella et al., 2004). While most bovine mastitis isolates appear to be *bap*-negative as the *bap* gene seems to be only present in a small percentage of bovine mastitis isolates, *bap* appears to be absent from human isolates (Cucarella et al., 2001). In the experiments in this example, although cell aggregation in the isogenic *bap* mutant strain M556 was much less than its parent V329, the data suggests that c-di-GMP treatment inhibits cell clumping in M556 (Fig. 6E amd 6F). This data from analyses of wildtype and mutant strains seems to imply that the inhibition of cell interactions is independent of *bap* and the *icaADBC* gene clusters. Importantly, the results of the microscopic analysis correlated with the macroscopic observations and further indicate that c-di-GMP treatment can

inhibit *S. aureus* cell-to-cell (intercellular) adhesive interactions in human and bovine isolates.

[00113] **c-di-GMP inhibits biofilm formation in human and bovine *S. aureus*.** Given that c-di-GMP treatment inhibits *S. aureus* cell-to-cell interactions, the present inventor predicts that c-di-GMP inhibits biofilm formation. The quantitative biofilm results showed that c-di-GMP treatment inhibits *S. aureus* DK825 biofilm formation on abiotic polystyrene surfaces at 24 h in a dose-dependent manner (Fig. 7A and 7B). The inhibitory effect of extracellular c-di-GMP was seen at 20 µM (~50% reduction), 200 µM (~65% reduction) and 400 µM (~85% reduction). A similar difference in biofilm formation between treated and untreated cultures was observed after measurement at 48 h suggesting that selection for resistance to treatment did not occur (data not shown). The results also showed similar c-di-GMP inhibition of biofilm formation at 24 h in the highly adherent hyperbiofilm *S. aureus* strain 15981 at the concentrations tested (Fig. 7C). Although coagulase has been shown to be important for *S. aureus* colonization to host tissues, no difference in coagulase production (bound or free) in DK825 between treated and untreated cells was found (data not shown). The quantitative biofilm results correlate with the macroscopic and microscopic cell-to-cell aggregation data suggesting that extracellular c-di-GMP inhibits cell interactions and biofilm formation in human isolates.

[00114] Quantitative biofilm analysis also demonstrated that c-di-GMP inhibits biofilm formation of bovine subclinical mastitis strains (Fig. 8A-8D). The wildtype bovine strain V329 was previously shown to be a strong biofilm producer on polystyrene surfaces while the isogenic *bap* mutant M556 was attenuated in this biofilm ability (Cucarella et al., 2001). The analysis here supports this finding but also importantly shows that like human

isolates, c-di-GMP dramatically inhibits biofilm formation (~50-
70% reduction) in these wildtype and mutant bovine strains as
well as in the wildtype *bap*-negative strain V299 and the wildtype
bap-negative *icaADBC*-negative strain V315 that formed very low
levels of biofilm (Figs. 8C and 8D). Together, these results
provide further compelling evidence to indicate that c-di-GMP
treatment inhibits *S. aureus* cell-to-cell interactions and cell-
to-surface interactions involved in biofilm formation. These
findings also suggest that cyclic dinucleotides such as c-di-GMP
would be useful in preventing biofilms on clinically relevant
surfaces such as medical devices and potentially in the control
of human and animal infection.

[00115] The regulatory mechanisms involved in *S. aureus* biofilm
formation are not fully understood. However, *S. aureus* biofilms
formation is known to be mediated through the production of the
extracellular polysaccharide intercellular adhesin (PIA/PNAG/PSA)
that is synthesized by the *icaADBC* genes and also has a role in
cell aggregation (Cramton et al., 1999; Maira-Litran et al., 2002
and McKenney et al., 1999). The SarA regulator has been shown to
be important for biofilm formation (Beenken et al., 2003; Blevins
et al., 2002 and Valle et al., 2003) as has the Bap protein
(Cucarella et al., 2001 and 2002). While the exact mechanism of
action remains to be determined, our results for strain 15981
suggest the mechanism might be *agr*-independent and our earlier
studies with the bovine isolates suggest that the mechanism might
be independent of *bap* and *icaADBC*.

[00116] **Effect of cGMP and 5'-GMP on biofilm formation.** Based
on the observed effects with c-di-GMP, the laboratory of the
present inventor then tested whether treating cultures with
extracellular guanosine nucleotide analogs such as cGMP
(guanosine 3', 5'-cyclic monophosphate) and 5'-GMP (guanosine 5'-
monophosphate) at the same concentration (200 µM) could also

inhibit *S. aureus* DK825 biofilm formation. These experiments were performed to rule out the possibility that the effects on biofilm observed in the studies in this example were merely due to the presence of extracellular guanosine nucleotides in general or cyclic guanosine (mononucleotide) analogs. These two particular nucleotides were also chosen as the structure of c-di-GMP is somewhat similar to two cGMP molecules being linked head-to-tail (3'-5') and 5'-GMP is a known breakdown product of c-di-GMP (Ross et al., 1991). The addition of cGMP to the growth medium was found to inhibit biofilm formation (~40%) but to a much lesser extent than c-di-GMP while 5'-GMP had no effect on biofilm formation compared to c-di-GMP (Figs. 9A and 9B). These findings indicate that the inhibitory effect observed with c-di-GMP compared to cGMP is not due to the molecule merely having a guanosine base or merely being cyclic in nature but is somehow unique to its cyclic dinucleotide structure. It is believed that the overall lack of potency of cGMP and 5'-GMP in their ability to inhibit biofilm formation in *S. aureus* compared to c-di-GMP further highlights the importance, novelty and perhaps specificity and affinity of c-di-GMP in its mechanism of action and effect on the cell. Considering the unusual shape and structure of the molecule, it is possible that it might bind with some specificity to a particular cell (or cell wall) target or receptor.

[00117] **Effect of c-di-GMP treatment on *S. aureus* pre-formed biofilms.** Since data presented in this example showed that c-di-GMP treatment inhibited biofilm formation in *S. aureus* strains DK825 and 15981, the hypothesis that extracellular c-di-GMP has an effect on pre-formed established biofilms was tested. The results obtained showed that c-di-GMP treatment (200 µM) of a 24 h pre-formed biofilm blocks further biofilm development (~75% reduction) compared to the untreated control (Fig. 10). Based on

these data, it appears that c-di-GMP inhibits both the initial formation of biofilms and the further development of pre-formed biofilms.

[00118] **c-di-GMP treatment inhibits *S. aureus* adherence to human epithelial cells.** Studies examining the adherence of *S. aureus* to epithelial cell monolayers and the effect of potential therapeutic agents to inhibit adherence have been performed (Balaban et al., 2003; Cucarella et al., 2002; Matsuura et al., 1996; Miyake et al., 1989 and 1991; Roche et al., 2003; and Wyatt et al., 1990). The data from the studies here showed that treatment with 2- and 20 μM c-di-GMP did not show any obvious effect on adherence. However, compared to untreated controls (Fig. 11A), treatment with 200 μM c-di-GMP reduced the numbers of *S. aureus* cells adhering to HeLa cells (Fig. 11B). The data indicated that c-di-GMP treatment results in an average reduction in adherence from 12 bacteria/cell to 4 bacteria/cell (~66% reduction). Experiments examining the effects of various concentrations (0-, 25-, 50-, 100-, 200- and 400 μM) of c-di-GMP on HeLa cells prepared as described above in complete media (but in the absence of bacteria) showed no obvious visible effect on HeLa cell morphology examined microscopically at 12-, 24- and 48 h incubation. While the molecular basis for c-di-GMP inhibiting *S. aureus* epithelial cell adherence is not yet understood, these *in vitro* data are clearly consistent with previous biofilm results using polystyrene (abiotic) surfaces and suggest that c-di-GMP can also be used to inhibit biofilm formation of epithelial cell (biotic) surfaces.

[00119] **Safety and Toxicity tests.** Analysis of HeLa cells showed that c-di-GMP treatment with concentrations up to 400 μM did not cause any obvious changes in cell morphology after 24 h exposure. However, HeLa cells treated with 400 μM c-di-GMP did appear to undergo morphological changes after 48 h exposure.

These findings suggest that c-di-GMP at concentrations <400 μM appear to be relatively noncytotoxic to these epithelial cells under the conditions tested.

[00120] The safety and potential lethality of c-di-GMP was further examined in CD-1 mice. In these studies, examination of the mice 24 h after c-di-GMP treatment (50 μL of 200 μM, orally) showed all mice were alive and no lethal effects were observed. Following sacrifice, various tissues and fluids were collected for future histological and biochemical analysis. Although the level of tissue distribution following c-di-GMP treatment and its potential tissue toxicity is not yet known, but is being currently studied, the *in vivo* studies show that c-di-GMP treatment at this concentration is nonlethal in mice and support other data from the laboratory of the present inventor indicating that c-di-GMP is relatively safe and nontoxic.

[00121] **Possible mechanism of action of c-di-GMP on *S. aureus*.** Studies in the Gram-negative bacterial species *A. xylinum* showed that c-di-GMP is an intracellular signaling molecule and does not appear able to enter these bacterial cells. Based on the findings here in *S. aureus*, a Gram-positive species, it is speculated that *S. aureus* can sense and repond to extracellular c-di-GMP. These findings suggest that extracellular c-di-GMP treatment and the resulting inhibition of cell-to-cell interactions and biofilm formation might involve c-di-GMP binding to a receptor (possibly exposed on the cell surface) which then triggers signaling events modulating gene and protein expression. As c-di-GMP has been reported to be able to enter eukaryotic cells (Steinberger et al., 1999), another possibility is that c-di-GMP might be able to enter *S. aureus* and trigger changes in protein expression. Regardless of the molecular mechanism involved, the findings in this example clearly indicate that c-di-GMP treatment inhibits cell-to-cell interactions and biofilm

formation in *S. aureus*. This ability would be a valuable
auxillary property to antimicrobial treatments as it might
increase the availability of the bacterial target site to the
antibiotic.

EXAMPLE 4

[00122] **Extracellular c-di-GMP regulates *V. cholerae* biofilm
formation.** Tests examining the stability of the molecule under
different buffers and temperatures have shown it is stable for at
least several months in physiological saline at 4°C and stable
following boiling for 10 min and stable following treatment in
acid (pH 3) and alkali (pH 10) for 1 h. Using a standard crystal
violet quantitative biofilm assay following 24 h static
incubation in glass test tubes containing LB broth supplemented
with various concentrations of c-di-GMP, extracellular c-di-GMP
was found to increase biofilm formation in *V. cholerae* N16961 in
a dose-dependent manner compared to untreated controls (Fig. 12).
These findings are consistent with findings in *G. xylinum* that
increasing c-di-GMP levels increases cellulose production. The
results obtained did not appear to be due to differences in
growth rate between treated and untreated cultures.
Interestingly, the RocS mutant shows less biofilm forming ability
compared to the wildtype strain and showed increased biofilm
forming activity in response to c-di-GMP. Given that RocS is
linked to c-di-GMP, the inability of extracellular c-di-GMP to
restore wildtype biofilm levels in the mutant could imply that
the RocS mutant is not only defective in c-di-GMP regulation but
in other properties associated with biofilm formation.

EXAMPLE 5

[00123] In a physiological environment, such as the surface of
a catheter, *S. aureus* is expected to colonize and form biofilms

enabling the persistence of cells under conditions of flow. To
mimic these conditions *in vitro*, and to further explore the
potential use of c-di-GMP as a novel anti-biofilm agent, a
continuous culture flow-cell biofilm model will be used to study
c-di-GMP treatment on *S. aureus* biofilm formation on silicon and
stainless steel surfaces. The effect of treatment with c-di-GMP
alone and treatment with c-di-GMP in combination with the
commonly used antibiotic oxacillin to increase susceptibility and
reduce biofilm formation on silicon and stainless steel will also
be tested.

**Flow-cell biofilm model to test the effect of c-di-GMP on *S.
aureus* biofilm formation**

i. Growth conditions

[00124] For biofilm cultures grown under dynamic flow on
silicon surfaces, the interior surfaces of 1 m sections of
silicon tubing (size 16, resulting volume of 7 ml, Masterflex)
will be used as attachment surfaces (see Fig. 13 for reactor
diagram). In experiments in which biofilms are to be grown
within a flow-cell, a Protofab Sealed Flowcell will be used (Fig.
14). This flow-cell can be incorporated into the middle of the 1
m sections of silicon tubing in the reactor system and has an
insert (either PMMA or stainless steel) that can be removed and
harvested for adherent biofilm. The hypothesis that c-di-GMP
treatment a) inhibits biofilm formation on silicon and steel; and
b) reduces pre-formed biofilms on silicon and steel will be
tested.

ii. Effect on biofilm formation

[00125] *S. aureus* DK825 cultures will be grown overnight at
37°C with shaking in TSB. The overnight culture is then diluted
1:1000 in fresh TSB and samples are grown with shaking at 37°C

until they have attained logarithmic growth phase. An aliquot (5
ml) of this logarithmic phase culture ($\approx 10^7$ CFU) containing 200
μM c-di-GMP (treated) and a sample inoculated with an identical
aliquot of 0.9% NaCl (untreated) will be injected into the tubing
and allowed to attach for 30 min before the flow of TSB (with c-
di-GMP and without) is started (0.7 ml/min). Since the residence
time is 7.5 minutes (less than the $\approx$30-40 minute bacterial
doubling time in batch growth conditions), only attached
organisms are retained on the surfaces. All biofilm experiments
will be conducted within a 37°C incubator and all samplings will
be performed in triplicate. Biofilm samples will be harvested 24
h post-inoculation by scraping the silicon and steel surfaces,
thereby loosening the biofilm. Each sample will be suspended in
2 ml PBS and vortexed to disrupt any aggregated cells. Serial
dilutions of the vortexed samples will be plated out onto blood
agar plates in order to enumerate cfu/ml of treated and untreated
samples and therefore the effect of c-di-GMP on the viability of
S. aureus in a model biofilm system on silicon and steel.

iii. Effect on pre-formed biofilms

[00126] *S. aureus* DK825 will be grown overnight at 37°C with
shaking in TSB. The overnight culture is then diluted 1:1000 in
fresh TSB and samples are grown with shaking at 37°C until they
have attained logarithmic growth phase. An aliquot (5 ml) of
this logarithmic phase culture ($\approx 10^7$ CFU) without c-di-GMP will
be injected into the tubing and allowed to attach for 30 min
before the flow of TSB either with 200 μM c-di-GMP (treated) or
containing an identical aliquot of 0.9% NaCl (untreated) is
started (0.7 ml/min). Since the residence time is 7.5 minutes
(less than the $\approx$30-40 minute bacterial doubling time in batch
growth conditions), only attached organisms are retained on the
surfaces. All biofilm experiments are conducted within a 37°C

incubator and all samplings will be performed in triplicate.
Biofilm samples will then be harvested 24 h post-inoculation by
scraping the silicon and steel surfaces, thereby loosening the
biofilm. Each sample will be suspended in 2 ml PBS and vortexed
to disrupt any aggregated cells. Serial dilutions of the
vortexed samples will be plated out onto blood agar plates in
order to enumerate cfu/ml of treated and untreated samples and
therefore the effect of c-di-GMP on the viability of *S. aureus* in
a model biofilm system on silicon and steel will be tested.
Since the results in Example 3 suggest that c-di-GMP treatment
reduces cell-to-cell interactions, cell-to-surface interactions
on plastic, epithelial cell adherence and reduces *S. aureus* cell
viability, the present inventor expects to find that c-di-GMP
will inhibit biofilm formation on silicon and steel and will also
reduce pre-formed biofilm levels.

**Flow-cell biofilm model to test the effect of c-di-GMP and
antibiotics on susceptibility and biofilm formation**

[00127] Based on preliminary data in the laboratory of the
present inventor, it is expected that c-di-GMP treatment
increases the antibiotic susceptibility of *S. aureus*. Therefore,
to further explore the potential use of c-di-GMP as an
"antibiofilm" agent, the hypothesis that c-di-GMP treatment
increases antibiotic susceptibility will be tested. Initially,
the effect of c-di-GMP alone and in combination with the commonly
used antibiotic oxacillin to increase the susceptibility of
biofilm-associated *S. aureus* will be tested. These studies will
use the continuous culture flow-cell biofilm model described
above to analyze *S. aureus* biofilm formation in the presence of
c-di-GMP alone and in combination.

[00128] To study the potential of any combined antibiotic
activity (synergy) of c-di-GMP and oxacillin against *S. aureus*
biofilms, a modification of the flow-cell biofilm model described

above will be used. The MIC of oxacillin will first be
determined using standard tube assays by testing concentrations
1-1,024 μg/ml using National Committee for Clinical Laboratory
Standards (NCCLS). The MIC of oxacillin in the presence of 200
μM c-di-GMP will be quantitated to determine if there is any
reduction in susceptibility and synergy.

[00129] For studies in the biofilm model, *S. aureus* DK825 will
be grown overnight at 37°C with shaking in TSB. The overnight
culture is then diluted 1:1000 in fresh TSB and samples are grown
with shaking at 37°C until they have attained logarithmic growth
phase. An aliquot (5 ml) of this logarithmic phase culture ($\approx 10^7$
CFU) containing i) 200 μM c-di-GMP alone, ii) c-di-GMP+oxacillin
(concentration determined above), iii) oxacillin alone, and iv)
0.9% NaCl alone will be injected into the tubing and allowed to
attach for 30 min before the flow of TSB (same conditions as
above) is started (0.7 ml/min). Since the residence time is 7.5
minutes (less than the $\approx$30-40 minute bacterial doubling time in
batch growth conditions), only attached organisms are retained on
the surfaces. All biofilm experiments are conducted within a
37°C incubator and all samplings will be performed in triplicate.
Biofilm samples will be harvested 24 h post-inoculation by
scraping the silicon surface, thereby loosening the biofilm.
Each sample will be suspended in 2 ml PBS and vortexed to disrupt
any aggregated cells. Serial dilutions of the vortexed samples
will be plated out onto blood agar plates in order to enumerate
cfu/ml of treated and untreated samples and therefore the effect
of c-di-GMP on the antibiotic susceptibility and inhibition of *S.
aureus* biofilm formation. Time-kill curves similar to the method
of Domaracki *et al.* (Domaracki et al., 2000) will also be
performed to study the effect of combinations of c-di-GMP and
oxacillin on the growth of MRSA strain DK825 throughout a 12-h
incubation in the biofilm model described above. The strain will

be tested in TSB plus 2% NaCl with a sub-MIC concentration of
oxacillin alone (concentration to be used will be determined
above) or in combination with c-di-GMP (200 μM). In these
studies, viability counts will be performed at 0, 3, 6, and 12 h
by plating samples from the flow-cell system onto blood agar.

[00130] The biofilm model system used in this example mimics
the clinical setting (e.g., a medical device) as a constant
concentration of the "drug" is being administered with the flow-
cell system and avoids re-administering drug at later intervals.
Since data in Exmaple 3 above suggests that c-di-GMP inhibits
cell viability by approximately 1-log and inhibits cell-to-cell
interactions and biofilms under the previous conditions tested,
reductions with c-di-GMP treatment in this model biofilm system
are expected. While it is possible, no antagonism to oxacillin
susceptibility is anticipated. Also, while unlikely, given the
inhibition of cell-to-cell interactions and thus the presumed
better access of antimicrobial targets, it is possible that c-di-
GMP will not be synergistic and will not increase the antibiotic
susceptibility of DK825. However, the present inventor expects
to find that combination c-di-GMP treatment will produce a
synergistic effect and cause a measurable increase in antibiotic
susceptibility in S. aureus compared to oxacillin or c-di-GMP
treatment alone. Such a synergistic effect would demonstrate
that c-di-GMP could be used in combination with other drugs to
increase antimicrobial activity. If differences in
susceptibility are found in this system, these findings would
support c-di-GMP having useful antimicrobial activity, and
further susceptibility studies will be performed using different
antibiotics. Time-kill curves are expected to show an
enhancement of killing in the presence of combinations of c-di-
GMP and oxacillin. Synergy would be indicated if, for example,
2-logs of killing were found compared to the starting inoculum

for *S. aureus*. This study might indicate that sub-MICs of
oxacillin together with c-di-GMP are effective in killing or at
least increasing the susceptibility of MRSA. If synergy is
found, further investigations could be conducted with animal
models to see if a c-di-GMP-oxacillin combination is synergistic
in vivo. Additional testing could also be performed to determine
the prevalence of a synergistic effect in different species of
staphylococci and in the clinical staphylococcal population.

EXAMPLE 6

[00131] The sequencing of the *V. cholerae* strain N16961
(Heidelberg et al., 2000) has enabled new approaches to studying
this pathogen. A microarray of the N16961 genome, represented by
3884 oligos (1 oligo/gene of the genome) was printed on
polylysine-coated glass microscope slides (Corning) using a
microarray maker, PS5200 (Cartesian Technologies). The
oligonucleotide probes were suspended in 20 mMTris, 50 mM KCl, pH
6.5, 50 % DMSO as a printing buffer, then arrayed in a 96 well
plate and spotted under appropriate conditions of temperature and
humidity. After printing, the slide is dried and then the
spotted DNA is bound to the slide by UV-crosslinking at 60 mJ
using a Stratalinker (Stratagene) and baked at 80 C for two
hours. Target nucleic acid will be labeled either with Cy3 or
Cy5 fluorescent dyes.

[00132] Proteins with a GGDEF domain have regulatory roles and
are thought to be associated with c-di-GMP. Since *V. cholerae*
contains approximately 41 GGDEF proteins (Galperin et al., 2001),
the present inventor expects that c-di-GMP regulates many genes
and important processes in *V. cholerae* (and other pathogens),
including virulence. This hypothesis will be tested using a
transcriptional profiling approach to study the transcriptome of
V. cholerae strain N16961.

[00133] In these initial studies wildtype cells grown in the
presence and absence of c-di-GMP will be compared to identify
genes whose expression is affected by c-di-GMP. RNA from log
phase cells grown in LB broth at 37°C shaking will be extracted
from the cultures after adjusting to OD_{600} 0.3-0.4 using RNA Easy
kit (Qiagen) and treated with DNase I (Qiagen) before labeling.
For labeling reactions, mRNA will be labeled either with Cy3 or
Cy5 dyes. Both labeling reactions will be performed with all
samples to account for systematic variation based on the labeling
reaction and characteristics of the dyes. The fluorescent label
will be incorporated using the standard method involving reverse
transcriptase with amino acyl-labeled nucleotides.
Unincorporated dNTPs and the oligo-dT primers will be removed by
ethanol precipitation. The label is incorporated in a second
step. Amino acyl labeling was found to provide a more uniformly
product from sample to sample in control samples. In order to
maximize the signal and minimize the background, the microarrays
will be prehybridized in 1 M NaCl, 50 mM Tris pH 7.0, 50%
formamide, 10% dextran sulfate, 1% SDS and 1% bovine serum
albumin for 1 hr and 42 C. The labeled target nucleic acid is
denatured at 95°C for 5 minutes, cooled to 4°C and added to the
hybridization chamber containing the slide. The hybridization
reaction incubates at 42°C overnight. The slide is removed from
the chamber and washed with 0.1 M NaCl and 0.1% SDS for 5 minutes
at room temperature. The slide is then allowed to air dry. The
hybridized microarrays will be scanned in a Scan Array 3000 (GSI
Lumonics) scanner. The scanner uses lasers operating at 633 nm
and 543 nm to excite Cy5 and Cy3 respectively. Cy5 is scanned
first because it is more sensitive to photodegradation. Data
from each fluorescent channel is stored separately as a TIFF
file. The Imagene (Biodiscovery) software will be used to
identify the location of each spot, link the spot identifiers,

measure the background density around each spot, and record the fluorescent intensities into a simple flat file. Data from replicate spots and from spots representing the same taxa will be compared, outliers discarded and the others averaged using the software GeneSight (Biodiscovery).

[00134] **Experimental Design of microarray data analysis.** To compare the expression profile of the cells grown with and without c-di-GMP, a Latin square design will be used. For each pair of samples (with and without c-di-GMP), two aliquots of each sample will be prepared, i.e., two from the sample without c-di-GMP (A1 and A2) and two from the sample with c-di-GMP (B1 and B2). The first array will consist of the first untreated sample (A1) labeled red and hybridized with the first treated sample (B1) labeled green. The second array will consist of the second treated sample (B2) labeled red and hybridized with the second untreated sample (A2) labeled green. The layout of the Latin square design is shown below in Table 4:

Table 4

Dye	Chip 1	Chip 2
Red	A1	A2
Green	B1	B2

[00135] This design will be very effective in eliminating of dye bias for each with- and without c-di-GMP comparison. Six chips will be used since the Latin square design will be applied for each triplicate pair of samples.

[00136] **Data Processing.** Typically, the background of a microarray image is not uniform over the entire array. The procedure of extraction of local background intensity will be implemented. The (background subtracted) intensity values from the two channels from each array will be plotted against one

another to check the quality of the data, namely that data for
all genes whose transcription levels have remained essentially
unchanged should fall on a straight diagonal line. The range of
the values should be nearly the same for all channels, and
neither channel should be saturated. Log transformation ensures
that the data are approximately normally distributed within each
gene, which improves statistical performance of the analysis of
variance. The method is fairly robust to departures from
normality. The log base2 scale will be used in our analysis
because each unit after transformation corresponds to a two-fold
difference.

[00137] **Normalization.** To account for experiment-wide
systematic effects that may bias inference made on the data from
the individual genes, an ANOVA model which fits the raw
fluorescence intensity values as a function of dye and array will
be used to perform a normalization (Wolfinger et al., 2001). Let
y_{gijk} be the base-2 logarithm of the background-corrected
intensity value from gene g, variety i $(i =1,2)$, dye j $(j=1,2)$
and array k $(k=1,*,6)$. The genetic term "variety" here signifies
the type of the mRNA samples (from N16961 wildtype or VpsR mutant
cells). The normalization model is:

$$y_{gijk} = \mu + D_j + A_k + (DA)_{ik} + \varepsilon_{gijk} . \qquad (1)$$

where μ is an overall mean, D is the main effect for dyes, A is
the main effect for arrays, DA is the interaction effect of
arrays and dyes, and ε is a random error. In model (1), the main
and interaction effects may be treated as random effects. The
residuals from this model, computed by subtracting the fitted
values for the effects from the y_{gijk} values can be viewed as the
relative fluorescence intensities for each gene relative to the
sample mean. The basic idea of the normalization model is to
remove overall differences between the dyes and between the
arrays.

[00138] **Gene-Specific Significance Models.** To identify
differentially expressed genes between treated and untreated
cells, perform gene-specific ANOVAs (Wolfinger et al., 2001) will
be performed. Let r_{gijk} denote the residuals from the model (1),
i.e., the relative fluorescence intensities for gene g. The gene
model is:

$$r_{gijk} = V_{gi} + D_{gj} + (VD)_{gij} + \varepsilon_{gijk} . \quad (2)$$

[00139] All effects are indexed by g and are assumed to serve
similar roles to those in model (1) but at the gene level, V is
the main effect for varieties. Except V, other effects may be
treated as random effects. The estimates of primary interest are
those of the V_{gi} effects, which measures the variety effects for
each gene. Differences between these effects will be tested by
using SAS procedure such as PROC MIXED within a gene. Based on
this statistical approach, the cut-off will be established.
Finally, a list of differentially expressed genes will be
selected for each comparison.

[00140] **Alternative Analysis.** The Significant Analysis of
Microarrays (SAM) (Tusher et al., 2001) is an alternative to the
ANOVA approach. SAM, developed by Rob Tibshirani, Stanford
University, is a statistical method adapted specifically for
microarrays. SAM assigns each gene with a score that is based on
its change in gene expression relative to the standard deviation
of repeated measurements for that gene. Genes with scores
greater than a threshold are deemed potentially significant.
This procedure provides the false discovery rate (FDR), the
percentage of such genes identified by chance. By controlling
proper FDR (such as 0.05), a set of differentially expressed
genes will be identified for each comparison. In comparison with
the results of ANOVA, more attention will be paid on those genes
identified by both methods.

[00141] **Expected results and interpretations.** These
transcriptional profiling studies will identify genes that are
regulated by c-di-GMP. Specific genes of interest (e.g.
regulatory and virulence) that are significantly activated or
repressed by c-di-GMP will be confirmed by RT-PCR and can be
further studied. Even if c-di-GMP regulates known virulence
genes, this would represent a novel signaling cascade controlling
virulence. Using a similar approach, the effect of c-di-GMP and
analogs in the regulation of other pathogens can be studied.

EXAMPLE 7

[00142] A *Staphylococcus aureus* amplicon-based microarray was
constructed by TIGR (The Institute for Genomic Research,
Rockville, MD) and contains amplicons representing segments of
2480 ORFs from *Staphylococcus aureus* strain COL (reference
strain), *Staphylococcus aureus* strain MU50, *Staphylococcus aureus*
strain MW2, and *Staphylococcus aureus* strain N315. The
microarray is also printed with control transcript spots.

[00143] Table 5 below show a subset of the microarray data
obtained on the effects of c-di-GMP (200 μM) on *S. aureus* strain
DK825. Only some of the known regulatory, virulence- and
biofilm-associated genes whose expression (level of
transcription) is differentially regulated (either up-
regulated or down-regulated) in response to c-di-GMP are shown in
this table. The fold-increase or decrease in expression for each
gene, as determined by the intensity of hybridization of the
amplicon spots on the microarray to RNA isolated from *S. aureus*
treated with 200 μM c-di-GMP compared with the intensity of
hybridization to RNA isolated from the *S. aureus* untreated
control, is presented.

TABLE 5

Up-regulated

agrA (quorum sensing)	5.4
agrB (quorum sensing)	5.8
agrC (quorum sensing)	2.0
agrD (quorum sensing)	2.2
saeR (response regulator)	2.5
saeS (histidine kinase)	1.5
rsbW (anti-sigmaB)	4.5
PBP-2	2.0
PBP-4	1.7

Down-regulated

fnbB (fibronectin binding)	0.47
fbnA (fibrinogen binding)	0.66
clfA (clumping factor)	0.54
clfB (clumping factor)	0.43
icaR (intercellular adhesin)	0.62
collagen adhesin	0.55
vacuolating cytotoxin	0.56
enterotoxin A	0.54
enterotoxin 1	0.35
exfoliative toxin	0.52
toxic shock syndrome toxin	0.45

[00144] The data indicate that c-di-GMP affects the expression of numerous genes in *S. aureus*. Notably, the c-di-GMP molecule affects expression of quorum sensing (agr) genes, regulators with known roles in virulence, toxin genes and colonization and biofilm-associated genes. The data are consistent with *S. aureus* being attenuated for biofilm formation, colonization, cell clumping, toxin activity and overall (regulation) of virulence. The rsbW gene encodes an anti-sigmaB factor that represses the expression of sigmaB which is important for full virulence. Therefore, increased expression of rsbW is consistent with decreased virulence.

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EXAMPLE 8

[00145] A mouse mastitis model of *S. aurreus* infection using the *S. aureus* Newbould 305 strain (Brouillette et al., 2003, 2004a and 2004b) was used to show the effectiveness of treatment with c-di-GMP. Three mice per group and two glands per mouse were used for a total of six samples per treatment. 100 CFU (colony forming units) of *S. aureus* Newbould 305 strain were inoculated into each mammary gland. Five or 50 nanomoles of c-di-GMP was administered by injection twice into each mammary gland at 0 hr. (pre-mixed with the *S. aureus* inoculum in 100 μl volume, i.e., 50 or 500 μM concentration) and at 4 hr. post-infection (in 50 μl, i.e., 100 or 1000 μM). The mammary glands were harvested at 10 hrs. post-infection. Considering the volume of the mammary gland with milk to be about 250 μl, the final concentration of c-di-GMP in the mammary gland at each injection was estimated to be 20 μM or 200 μM. Treatment with c-di-GMP clearly show a significant dose-dependent suppressing effect (reduction in CFU counts) of c-di-GMP on the ability of *S. aureus* to multiply or colonize in the mammary gland (Fig. 15). The results show that 50 nanomoles of c-di-GMP injected into the mammary gland *in vivo* significantly inhibits *S. aureus* infection of the mammary gland by at least 10-fold (T test: p=0.004; Mann-Whitney U-test: p=0.009).

[00146] Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

[00147] While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the

inventions following, in general, the principles of the invention
and including such departures from the present disclosure as come
within known or customary practice within the art to which the
invention pertains and as may be applied to the essential
features hereinbefore set forth as follows in the scope of the
appended claims .

[00148] All references cited herein, including journal articles
or abstracts, published or corresponding U.S. or foreign patent
applications, issued U.S. or foreign patents, or any other
references, are entirely incorporated by reference herein,
including all data, tables, figures, and text presented in the
cited references. Additionally, the entire contents of the
references cited within the references cited herein are also
entirely incorporated by references.

[00149] Reference to known method steps, conventional methods
steps, known methods or conventional methods is not in any way an
admission that any aspect, description or embodiment of the
present invention is disclosed, taught or suggested in the
relevant art.

[00150] The foregoing description of the specific embodiments
will so fully reveal the general nature of the invention that
others can, by applying knowledge within the skill of the art
(including the contents of the references cited herein), readily
modify and/or adapt for various applications such specific
embodiments, without undue experimentation, without departing
from the general concept of the present invention. Therefore,
such adaptations and modifications are intended to be within the
meaning and range of equivalents of the disclosed embodiments,
based on the teaching and guidance presented herein. It is to be
understood that the phraseology or terminology herein is for the
purpose of description and not of limitation, such that the
terminology or phraseology of the present specification is to be

interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

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